

Studies on Host Responses to *Aphanomyces invadans*

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Dedicated to my parents

Declaration

This thesis has been composed by myself and is the result of my own investigation. It has not been submitted for any other degrees.

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Abstract

Aphanomyces invadans is the pathogen that causes epizootic ulcerative syndrome (EUS), an economically devastating fish disease in southern Asia. The present thesis considered possible improvements to current methods of monitoring EUS, and examined the mechanisms of the host immune response to *A. invadans* in order to establish whether they could be enhanced to reduce the impact of EUS on aquaculture.

Monoclonal antibody (MAb) technology was considered as a possible improvement to the histopathological methods currently used in diagnosis of EUS. Five MAbs were raised to day-old *A. invadans* germlings. Four gave weak reactions to *A. invadans* and cross-reacted with other *Aphanomyces* spp, though they may be useful for future studies on *A. invadans*. The other, designated MAb 3gJC9, only cross-reacted with the crayfish plague pathogen, *A. astaci*, and was used for the development of an immunohistochemistry protocol that may be of use in diagnosis.

Immunohistochemistry with MAb 3gJC9, which recognised an extracellular product (ECP) of *A. invadans*, was specific to *A. invadans* in fish tissue, although it also recognised *A. astaci* in plague-infected crayfish. It also recognised the mycelium in fish infected with ulcerative mycosis, indicating that ulcerative mycosis is synonymous with EUS. Preliminary observations indicated that both ECPs and what appeared to be a hitherto unreported early stage of the mycelium are important in the pathology of EUS.

Studies *in vitro* on the macrophages of EUS-susceptible giant gourami *Osphronemus gouramy* and silver barb *Barbodes gonionotus*, and EUS-resistant Nile tilapia *Oreochromis niloticus*, found that their macrophages were able to inhibit the growth of *A. invadans*. The macrophages of striped snakehead *Channa striata* did not inhibit *A. invadans*, which may account for their high EUS-susceptibility, especially as *A. invadans* strongly inhibited the respiratory burst of snakehead macrophages.

Studies on humoral immune responses revealed that complement inhibited *A. invadans* in the case of snakeheads, gourami and barbs but not tilapia or swamp eels *Monopterus albus*. The humoral responses of the latter were very different to the four other species, and not elucidated.

Low levels of anti *A. invadans* antibodies were found in tilapia and gourami from an EUS-endemic region, and high levels in snakehead. Snakehead antibodies appeared to be able to inhibit *A. invadans* even when complement was removed, but lower levels were produced at the low temperatures typically associated with EUS.

A range of potential immunostimulants were screened for the ability to enhance resistance to EUS. The two successful products were administered as feed supplements to snakeheads and barbs that were subsequently injected intramuscularly with *A. invadans*. One, the algal extract Ergosan, showed some beneficial effects on snakeheads although the challenge was inconclusive. The other, the vitamin supplement Salar-bec, accelerated the cellular immune response and reduced mortality in snakeheads and barbs, and enhanced antibody production in snakeheads.

The antibody response of snakeheads was further studied by comparing the anti-*A. invadans* antibody level, inhibitory activity of sera *in vitro* and protective capacity of sera from EUS-naïve snakeheads to that of snakeheads recently exposed to EUS and those subject to long term EUS-exposure. Sera of populations recently exposed to EUS showed an increased level of antibodies, but little improvement in inhibitory or protective activity. Sera from snakeheads that had endured long term exposure showed a wide range of antibody levels, but marked increases in inhibitory and protective activity. Antibodies cross-reacted with non-pathogenic *Aphanomyces* spp. in all cases.

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Abbreviations

AAHRI	Aquatic Animal Health Research Institute (Bangkok, Thailand)
ACIAR	Australian Centre for International Agricultural Research (Canberra, Australia)
ACP	Alternative complement pathway
AVL	Aquaculture Vaccines Ltd. (Saffron Walden, UK)
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APES	3- aminopropyltriethoxysilane
APW	Autoclaved pond water
ATCC	American Tissue Culture Collection
BFAR	Bureau of Fisheries and Aquatic Resources (Manila, Philippines)
BSA	Bovine serum albumin
CP	Charoen Pokphand (Bangkok, Thailand)
df	Degrees of freedom
DAB	3,3' diamaminobenzidine
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
ECP	Extracellular product
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EUS	Epizootic ulcerative syndrome
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GMS	Grocott's methenamine silver
GPY	Glucose peptone yeast
H&E	Haematoxylin and eosin
HAT	Hypoxanthine aminopterin thymidine media supplement
HRP	Horse radish peroxidase
IFAT	Immunofluorescence antibody technique
Ig	Immunoglobulin
IHC	Immunohistochemistry
LSW	Low salt wash buffer
MAb	Monoclonal antibody
MG	Mycotic granulomatosis
MGC	Multinucleate giant cell
MRC	Multiple reaction cycling
MW	Molecular weight
NAGRI	National Genetics Research Institute (Patum Thani, Thailand)
NBT	Nitro blue tetrazolium
NCP	Nitro-cellulose paper
NIFI	National Inland Fisheries Institute (Bangkok, Thailand)
OIE	Office Internationale des Epizooties
PAb	Polyclonal antibody
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PG-1	Peptone glucose media one

PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid];1,4-Piperazinediethanesulphonic acid
PMA	4- α -phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonylfluoride
PRP	Phosphorylcholine reactive protein
RAC	Relative antibody concentration
RAPD	Random amplification of polymorphic DNA
Rf	Relative electrophoretic migration
RFLP	Restriction fragment length polymorphism
RPS	Relative percent survival
RSD	Red spot disease
sd	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAT	Tyramine amplification technique
TBS	Trizma buffered saline
TBSX	Triton X-100 supplemented trizma buffered saline
TMB	3'3'5'5'-Tetramethylbenzidine dihydrochloride
TTBS	Tween supplemented trizma buffered saline
UM	Ulcerative mycosis
UV	Ultra-violet
v/v	Volume by volume
w/v	Weight by volume

Publications

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1 General Introduction

1.1 Epizootic Ulcerative Syndrome

Since epizootic ulcerative syndrome (EUS) was discovered thirty years ago (Egusa & Masuda 1971), a considerable amount of literature has been published on the subject. There has been some controversy over the aetiology of EUS, although it is now rare for any researcher to attribute it to anything other than *Aphanomyces invadans*. In addition, the unusual severity of EUS has attracted much attention, and considerable effort has been expended on the diagnosis, monitoring and control of EUS.

This section presents a brief review of the available literature covering the causes and consequences of EUS.

1.1.1 History of Studies

The study of the disease now called epizootic ulcerative syndrome began in a freshwater farm stocked with ayu *Plecoglossus altivelis* in Oita prefecture, Kyushu, Japan in 1971. Over half the fish were infected and large numbers died. Egusa & Masuda (1971) reported the presence of a saprolegniacean oomycete (Section 1.2.1) of the genus *Aphanomyces*. The pathogen in question infiltrated the muscle tissue causing massive necrosis and raising ulcers on the surface of the fish.

The pathogen was later named *Aphanomyces piscicida* and the disease described as mycotic granulomatosis (MG) in several further Japanese language papers summarised by Egusa (1992) in English. After 1971, MG spread rapidly across Kyushu and on to neighbouring Honshu, causing similar fishkills in a wide range of species (Egusa 1992).

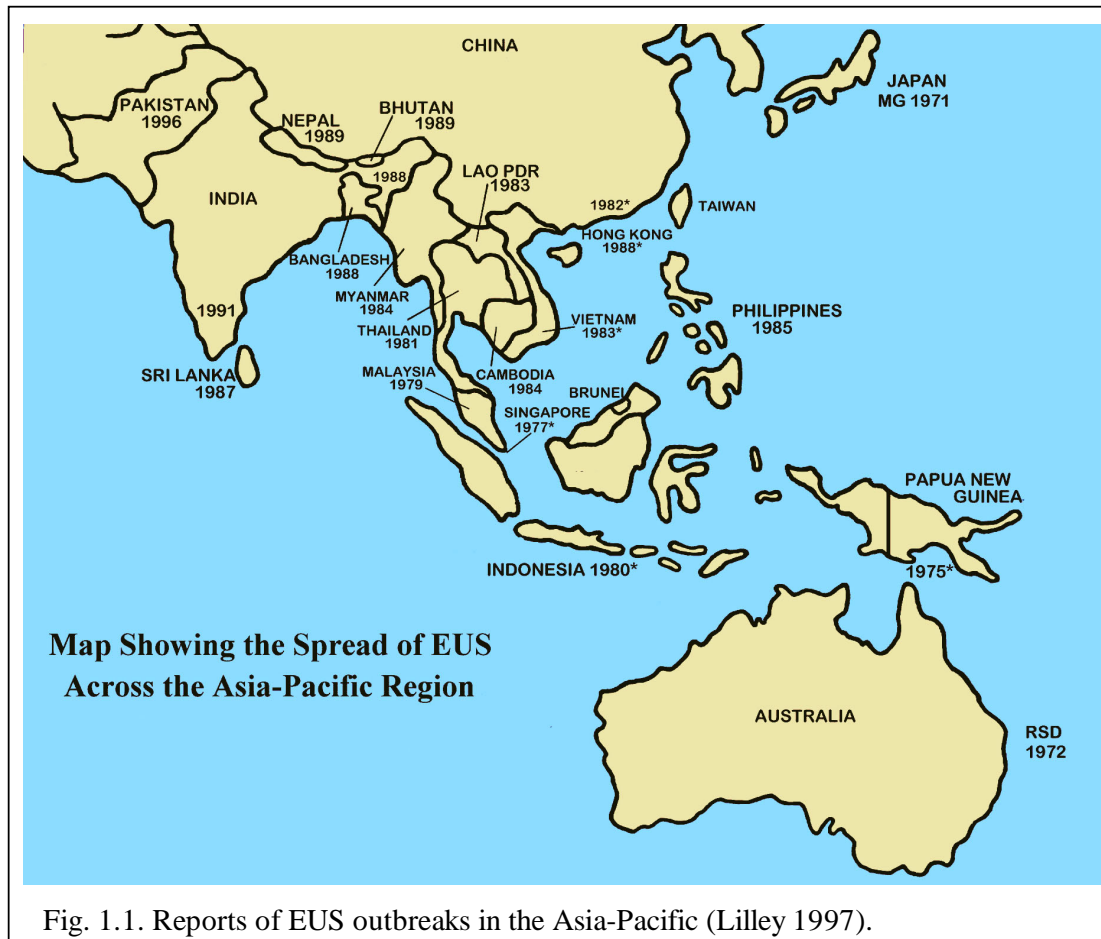


Fig. 1.1. Reports of EUS outbreaks in the Asia-Pacific (Lilley 1997).

In 1972, a similar pathogen was reported from Queensland, Australia associated with so-called red spot disease (RSD) of estuarine mullet *Mugil cephalus* (McKenzie & Hall 1976). RSD involved a saprolegniacean, which infiltrated muscle tissue, causing similar symptoms to MG though neither research group identified the similarities at the time.

With the benefit of hindsight, the spread of the disease now called epizootic ulcerative syndrome (EUS) across south east Asia is clear (Fig 1.1). However, the appearance of massive mortalities of ulcerated fish in Malaysia and Thailand in 1981 was again treated as a novel disease and aetiology was not immediately established. Tonguthai's (1985) summary of Thai research on the disease describes similar clinical signs from a wide range of species from a large geographical area. It also reports the

presence of many bacterial, parasitic and viral pathogens (Section 1.1.2) as well as the fungus.

Meanwhile, EUS spread throughout south east Asia and through Bangladesh to northern India and Nepal (Lilley *et al.* 1998). It reached the western limit of its current range in Pakistan, which it reached in 1996 (Chinabut 1998).

Roberts *et al.* (1993) identified the presence of an invasive *Aphanomyces* as a common factor in EUS-infected fish, and showed that similar pathology could be caused by hyphae isolated from such fish. The presence of ‘an invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response’ (Roberts *et al.* 1994) was later accepted as the case definition of EUS.

The *Aphanomyces* in question was formally described as *A. invaderis* (Willoughby *et al.* 1995), later changed to *A. invadans* for grammatical reasons (David & Kirk 1997). Lilley *et al.* (1997c) confirmed that the pathogen isolated from EUS was identical to that isolated from RSD and MG infected fish. Several studies established the pathogenic nature of the oomycete by inoculation of fish with mycelium (Hatai & Egusa 1979) and zoospores (Wada *et al.* 1996, Lilley & Roberts 1997). However, *A. invadans* could not be confirmed as the causative agent of EUS until Callinan (1997) reproduced an EUS outbreak by introducing zoospores into tank water.

In 1984, large scale fishkills characteristic of a new EUS outbreak occurred in the River Pamlico, South Carolina, USA. Histopathological analysis of infected fish revealed invasive hyphae identical to *A. invadans*, and the disease was subsequently called ulcerative mycosis (UM) (Noga & Dykstra 1986, Dykstra *et al.* 1986, Noga *et al.* 1988). Several oomycetes including *Aphanomyces* spp. and *Saprolegnia* spp. were isolated. While no isolate was able to induce the disease, lesion tissue itself did (Noga

1993a). Noga *et al.* (1996) concluded that the primary cause of UM was not a fungus or oomycete but the toxic dinoflagellate *Pfiesteria piscicida*.

Environmental stress factors are commonly associated with major EUS outbreaks (Section 1.1.4), and Kane *et al.* (2000) suggested that algal toxins may predispose fish to infection by a pathogen in the case of UM. A fresh outbreak of fishkills attracted further research, and an *Aphanomyces* apparently identical to *A. invadans* was isolated from a menhaden *Brevoortia tyrannus*, leading Blazer *et al.* (1999) to conclude that UM is indeed synonymous with EUS.

1.1.2 Pathogens other than *Aphanomyces invadans* involved in EUS

While *A. invadans* is now regarded as the necessary cause of EUS, many other pathogens have been associated with EUS lesions. Egusa & Masuda's (1971) original paper described the presence of unspecified metacercariae in infected ayu, and Tonguthai (1985) listed several other parasites involved in Thai outbreaks. No single parasite has been isolated from a substantial proportion of cases, so no parasite can be regarded as a necessary cause of EUS. However, Khan *et al.* (submitted) found that the presence of ectoparasites in a pond increased the risk of fish contracting EUS.

EUS frequently occurs in anoxic, eutrophic waterways with a rich microbiota, so it is unremarkable that a large number of bacteria have been associated with EUS infected fish. The ubiquitous *Aeromonas hydrophila* is commonly associated with EUS, but the diversity of pathogenic and non-pathogenic strains isolated from EUS lesions (Lio-Po *et al.* 1992) suggests that it is present as an opportunist rather than a primary pathogen. The clinical signs of EUS may be induced by injection of high concentrations of *A. hydrophila* in several species, but not by bath challenge as would be expected if *A. hydrophila* were a sufficient cause of EUS (Lio-Po *et al.* 1992, 1998). However, the fact that EUS-associated *A. hydrophila* is pathogenic at all suggests that it may contribute to

the pathology of EUS, and Boonyaratpalin (1989) suggested that it may be the cause of death in some cases.

Several other bacteria have been associated with EUS and particular attention was given to *Vibrio anguillarum* (Rodgers & Burke 1981) and an unclassified bacillus similar to the pathogen of human leprosy (Dastidar & Chakrabarty 1992, Mukherjee *et al.* 1995), but aetiology could not be established.

Roberts *et al.* (1990) regarded the spread of EUS as typical of an emergent virus. One rhabdovirus was found in outbreaks across Thailand and Myanmar, leading to speculation that it may have been the primary pathogen of EUS (Frerichs *et al.* 1986). However, extensive surveys of EUS-infected fish in Bangladesh did not find the same virus (Roberts *et al.* 1989) and later studies found that it was unable to induce pathology even by injection challenge (Frerichs *et al.* 1993).

A wide range of birnaviruses, reoviruses and rhabdoviruses have been isolated from EUS infected fish, but no one virus has been isolated from more than 5% of cases (Frerichs 1995). Kanchanakhan (1996) found that viral infections predispose fish to infection by *A. invadans*, but there is no evidence that any virus is a necessary cause of EUS.

Many oomycetes other than *A. invadans* are commonly isolated from EUS lesions, especially *Saprolegnia* spp. and *Achlya* spp. (Tonguthai 1985). However, such species are as omnipresent as *A. hydrophila* (Srivastava 1980, Ogbonna & Alabi 1991) and usually only found on the surface of the lesions (Roberts *et al.* 1993). They appear to be opportunistic saprophytes (Section 1.4.1) rather than primary pathogens, though they probably contribute to pathology once they are established.

1.1.3 Economic and Social Impacts of EUS

There is little quantitative data on the effect of EUS on the farmers and fishers of endemic regions as much of the impact falls on small scale farmers and local markets (Lilley *et al.* in press). A further difficulty is that the people directly affected by the disease are not always anxious to attract attention to it. For example, fishers in Laguna de Bay, Luzon, Philippines, attempted to conceal the first outbreaks from the public for fear that demand for fish would be affected (Palisoc & Aralar 1990).

Bhaumik *et al.* (1991) surveyed a region of West Bengal, India, recently affected by EUS. They found that 368 of the 500 small scale farmers involved in the study reported a loss of income of over 30% due to EUS, and a small number were forced to find alternative employment. Consumption of fish fell by 20-30%, which may have serious nutritional consequences in areas where fish is a major source of dietary protein.

It is not clear whether the severe impact reported in West Bengal are typical of new EUS outbreaks as there are wide variations in cultural preferences and economic status of people across the range of *A. invadans*. However, there is strong anecdotal evidence that the appearance of EUS was a strong disincentive to development of aquaculture systems in Nepal (Lilley *et al.* in press).

McAndrew (in press) found that the management practices on cage farms in Bangladesh have been adapted to minimise the impact of EUS. Any fish showing lesions was immediately removed from the system and usually consumed within the farmers' household. While annual losses to EUS in Bangladesh are now considerably below that of the US\$2,800,000 reported from the first outbreaks in 1988 (Lilley *et al.* 1998), it evidently remains a constraint to small scale aquaculture.

The fact that EUS may cause significant losses among wild populations extends its socially destructive effects to fishers. Studies of the Laguna region of Luzon revealed that EUS first appeared in 1986, but was still the most important factor in determining fish catch in 1992, although EUS-related losses peaked at 50% in 1989 (Lilley *et al.* in press).

Recent events in the southern Philippines have illustrated that the effects of EUS can extend beyond the purely economic in areas of political tension. In western Mindanao, Philippines, the predominantly Moslem fishing community has been badly affected by recent outbreaks of EUS, and blame predominantly Christian African catfish *Clarias gariepinus* farmers for introducing it by injudicious imports of stock from EUS-endemic regions. There is no objective data on the situation but there is strong anecdotal evidence that EUS has exacerbated existing ethnic tensions, and is at least partly responsible for acts of violence (from discussions with local workers).

1.1.4 Epidemiology of EUS

While the presence of *A. invadans* is regarded as the necessary cause of EUS, particular environmental factors are usually required to induce an EUS outbreak. Lilley *et al.* (1998) described three factors necessary for experimental induction of EUS, namely the presence of *A. invadans*, the presence of susceptible fish and exposure of the fish dermis.

The presence of *A. invadans* is possibly the least complex of the three factors, and the geographical spread of EUS (Fig. 1.1) is probably due largely to the spread of *A. invadans* itself. Its spread across the Asian mainland was probably facilitated by seasonal flooding caused by the annual monsoon, as the sudden appearance of EUS in large areas of Bangladesh, India and Nepal in the winter of 1987-8 was preceded by catastrophic flooding (Lilley *et al.* 1998).

The ability of EUS to cross sea boundaries requires more explanation as *A. invadans* cannot survive marine salinity (Fraser *et al.* 1992). The large scale, mainly unregulated trade in fish around Asia is the most likely mechanism of transmission, and *A. invadans* has been isolated from fish imported from Singapore to Japan (Wada *et al.* 1994). Further evidence for such a mechanism may be derived from the observation that the first outbreaks reported in Sri Lanka occurred in ponds near Colombo international airport through which many fish would have passed (Balasuriya 1994).

Once *A. invadans* has appeared in a region, a great many species are at risk. Lilley *et al.* (2001) listed 68 species which have been observed to be naturally infected by *A. invadans*. It is likely that a great many more species that are less common or less commercially important are also susceptible but have escaped the notice of researchers and health inspectors.

There appear to be degrees of susceptibility to EUS. Some species are often reported as being particularly susceptible such as the striped snakehead *Channa striata*, silver barb *Barbodes* (= *Puntius*) *gonionotus* and swamp eel *Monopterus* (= *Fluta*) *albus*, (Tonguthai 1985, Bhaumik *et al.* 1991, Vishwanath *et al.* 1997). Some species are susceptible in the colder parts of their range, but do not show symptoms in lower latitudes; for example the rohu *Labeo rohita* is reported as susceptible in China (Noga 1990) and Bangladesh (Lilley & Roberts 1997) but not in Southern India (Mohan & Shankar 1994). Other species have never been infected in the wild (Egusa 1992), and some appear to be resistant even to injection challenge, including the common carp *Cyprinus carpio* (Wada *et al.* 1996) and Nile tilapia *Oreochromis niloticus* (Khan *et al.* 1998).

Most EUS outbreaks are associated with environmental stress factors, and the presence of *A. invadans* alone is rarely sufficient cause for an EUS outbreak (Lilley *et*

al. 1998). The most widely reported environmental factor is temperature. Outbreaks have been associated with the colder seasons of the year by separate studies in Thailand (Tonguthai 1985), India (Vishwanath *et al.* 1997, Vishwanath *et al.* 1998) and North America, assuming that UM and EUS are the same disease (Dykstra *et al.* 1986). It is unclear why such seasonality should occur as *A. invadans* grows well in culture up to 30°C (Willoughby *et al.* 1995). The fact that the successive outbreaks in a region tend to become less severe and of shorter duration (Lilley *et al.* 1992) suggests that fish populations may develop resistance to EUS. Chinabut *et al.* (1995) showed that snakeheads from an EUS-endemic region, which are highly susceptible at 25°C, mount an effective immune response to injection challenge at 31°C, which suggests that wild fish would only be susceptible during the cold season. EUS may disappear completely from a region for several seasons, but reappear at a later date (Chinabut 1998).

Heavy rainfall is often associated with particularly severe outbreaks. The rapid advance of *A. invadans* across the Indian sub-continent after the 1987 flooding has already been mentioned. Studies in Australia and Thailand have associated sudden drops in temperature, oxygen and pH of river water caused by heavy rainfall with outbreaks of EUS (Rodgers & Burke 1981, Tonguthai 1985, Callinan *et al.* 1989, Keddie 1990). Low pH, due to acid sulphate soil runoff after heavy rainfall, has been shown to be a cause of dermatitis in fish, which allows attachment and invasion of the dermis by *A. invadans* (Sammut *et al.* 1996, Callinan 1997). Heavy rainfall may also reduce salinity in estuarine habitats to the extent that EUS may sporulate, and also cause considerable stress to estuarine fish. The combination of these effects may explain how marine species such as mullet and sand whiting *Sillago ciliata* have been infected with a pathogen that can only cope with fairly low salinity (Fraser *et al.* 1992).

Reduced hardness is another possible result of heavy rainfall that has been associated with outbreaks (Barua 1994, Das 1994).

Various stress factors not associated with rainfall have also been implicated, such as heavy metal pollution (Phillips 1994) or poor husbandry of pond farms leading to poor water quality (Barua 1994). Similarly, Khan *et al.* (submitted) found that high concentrations of organic debris increased the risk of EUS in pond farms by a factor of three.

While most reports of EUS describe seasonal epizootics and massive fishkills, recent studies in Bangladesh found a high prevalence of *A. invadans* in areas where no large scale mortalities took place, suggesting that it was functioning as a chronic pathogen (Lilley *et al.* in press). The same study found that prevalence of EUS was far less seasonal than most other reports suggest. No previous study had addressed the possibility that fish without major clinical signs may have EUS outside the colder seasons, but it is apparent that EUS may continue to infect fish and probably reduce farm productivity after it has apparently disappeared from a region. Such an observation may also explain the fact that EUS often reappears to cause fishkills in regions where it has not been observed for several years (Chinabut 1998).

1.1.5 Diagnosis of EUS

Unambiguous identification of EUS is essential for any attempt to regulate or control it. The case definition is ‘the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response’ (Roberts *et al.* 1994), and the Office Internationale des Epizooties (OIE) has designated the Aquatic Animal Health Research Institute (AAHRI), Thailand, as the reference laboratory for EUS.

The case definition of EUS can only be assessed histopathologically (Section 2.2) and much confusion has been caused by attempts to describe EUS on the basis of

clinical signs. For example, an EUS outbreak was described in South Vietnam in 1973, six years before any other report in Asia (Pham 1994), but no histopathology was performed so it is unclear whether the disease was actually EUS or another ulcerative condition.

No histopathological stain is able to distinguish *A. invadans* from other hyphal pathogens, and a more positive identification may be obtained by isolating *A. invadans* from infected tissue and identifying it by its growth rate, pathogenicity and the morphology of its sporangia (Lilley *et al.* 1998). Isolation has the disadvantage of being time consuming, and it is not always successful.

Although the progress of *A. invadans* infection may vary between species, Viswanath *et al.* (1997) were able to identify three stages of EUS lesions that are common to most occurrences (Section 1.3.1), from pinhead sized haemorrhages to large lesions that may expose muscular tissue and even the skeletal structure.

1.1.6 Control of EUS

Good husbandry remains essential for maintenance of good aquaculture stocks, and contributes to reducing the incidence of EUS. Nevertheless, several measures have been proposed specifically to counter the threat of EUS.

The first is simply to prevent *A. invadans* from reaching a region where it may cause outbreaks. The potential for the rapid spread of EUS by the international market in fish has been recognised for some years (Lilley *et al.* 1992), but new outbreaks of EUS continue to appear in areas that *A. invadans* could not have reached without human assistance (Fig. 1.1). For example, African catfish continue to be moved around the Philippines in untreated water in spite of advice to the contrary, which may have led to the recent outbreaks in Mindanao in 1998 and Panay in 1999 (BFAR, unpublished data).

Once EUS has appeared in a region, little can be done to prevent mortalities among wild stocks. However, farmers may still exclude *A. invadans* by careful selection of seedstock from EUS-free locations or well-monitored hatcheries, and by treating any introduced stock by immersion in 1% w/v sodium chloride (Lilley *et al.* 1998). Khan *et al.* (submitted) found that the presence of wild fish in pond farms gave the greatest risk of EUS out of a large number of factors investigated, so exclusion of wild fish should be regarded as a priority. The culture of non-susceptible species such as Chinese carps, tilapia or milkfish *Chanos chanos* is recommended at least for the period of the initial, most destructive outbreaks (Lilley *et al.* 1998), BFAR unpublished data). Where susceptible fish are farmed in EUS-endemic areas, fish showing clinical signs are often removed from culture systems immediately, as they act as reservoirs of infection (McAndrew in press).

The potential for EUS outbreaks in ponds is reduced where ponds are drained and mud removed from the bottom (Khan *et al.* submitted), and although not always practical, this is advisable before restocking ponds where EUS has already occurred.

Campbell *et al.* (2001) tested a number of substances used by aquaculturists in Southern Asia, and found that quicklime (CaO) and slaked lime (Ca[OH]₂) inhibited *A. invadans in vitro* at the concentrations at which they are widely used on farms. Other products such as wood ash and agricultural lime (CaCO₃) showed no inhibitory activity, but may stabilise the chemistry of ponds. They also found that the extract of the seeds of the neem tree *Azadirachta siamensis*, which is common throughout tropical Asia, showed inhibitory activity, although the leaves are more widely used to treat ponds.

Lilley & Inglis (1997) and Campbell *et al.* (2001) found that malachite green was strongly inhibitory *in vitro* and it has been used successfully in practice, though at the expense of palatability of treated fish (Shrestha 1994). However, malachite green

has been shown not only to cause adverse effects among the fry of some species, but to have teratogenic effects in mammals (Meyers & Jorgenson 1983) so its widespread use in fish farmed for food is not to be recommended. A product of unknown constitution called CIFAX is widely marketed in India, and Das & Das (1993) claim that it cures EUS at a concentration of 11 hectare-metre $(10,000\text{m}^3)^{-1}$, though no inhibitory activity was demonstrated at such concentrations *in vitro* (Campbell *et al.* 2001).

Tetracycline cured ulcerated *Puntius* spp. in pond trials (Roberts *et al.* 1989) and Lilley & Inglis (1997) found that antibiotics such as streptomycin and oxolinic acid inhibited *A. invadans* *in vitro*. However Lilley *et al.* (1992) reported that in practice, antibiotics are prohibitively expensive and mainly effective against secondary bacterial infections rather than *A. invadans* itself. Use of antibiotics in aquaculture systems may have serious consequences for human health as much of the antibiotic is likely to be released into the environment, where it promotes the spread of antibiotic resistance plasmids (Takashima *et al.* 1985, Aoki *et al.* 1987), which may spread to human pathogens.

1.2 The Biology of *Aphanomyces invadans*

Since EUS was recognised as an oomycosis, *A. invadans* has been extensively compared with other oomycetes to find its taxonomic position within the group, and its differences and similarities to related species.

The present section summarises the conclusions drawn from such studies, and compares them to other studies of oomycete biology.

1.2.1 Taxonomy

Although the first description of the EUS pathogen referred to it as *A. piscicida* (Hatai 1980), the name was subsequently rejected in favour of a more formal description

calling it *A. invaderis* (Willoughby *et al.* 1995), which was later modified to *A. invadans* for grammatical reasons (David & Kirk 1997).

Rapid amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) showed that *A. piscicida* and *A. invadans* are the same species, and show remarkable genetic homogeneity across the geographical range of the species (Lilley *et al.* 1997*b*). The homogeneity extends to the phenotype, as evidenced by the very similar carbohydrate profiles of isolates from across the range (Lilley *et al.* 1997*c*). No sexual stage has ever been reported from *A. invadans*, which may explain the lack of diversity within the species.

Willoughby *et al.* (1995) were able to place the pathogen in the genus *Aphanomyces* by the morphology of the zoosporangia (Section 1.2.2). Interestingly, RFLP analysis of various *Aphanomyces* spp. placed *A. invadans* very close to the other major animal pathogen in the genus, *A. astaci* (Hart 1998, Section 1.4.4).

A. invadans itself has not been used in any major study of the position of the genus *Aphanomyces* within the oomycetes. However, studies on ribosomal (Dick *et al.* 1999, Reithmüller *et al.* 1999) and mitochondrial (Hudspeth *et al.* 2000) DNA of the oomycetes place the genus *Aphanomyces* in the sub-class Saprolegniomycetidae, order Saprolegniales, family Saprolegniaceae (Dick 1999). The most closely related genus is *Leptolegnia*, which also includes several animal pathogens. Order Saprolegniales includes the genera *Achlya* (Section 1.4.1) and *Saprolegnia* (Sections 1.4.2, 1.4.3), members of which are commonly associated with fish diseases.

It has been suggested that the oomycetes should be reclassified as the taxon, Peronosporomycetidae (Dick 1995). In the absence of a definitive classification, the term ‘oomycete’ will be used throughout the present thesis as it is the term most frequently used in studies of fish diseases.

1.2.2 Life Cycle

The life cycle stages of *A. invadans* are typical of an oomycete (Dick 1999), other than the apparent lack of a sexual stage.

The aseptate mycelium is the stage found in infected fish. It is relatively wide in fish at 6-27µm, but considerably narrower in culture (Roberts *et al.* 1993). Asexual propagation in *Aphanomyces* spp. is carried out by the formation of zoosporangia at which primary zoospores cluster. The primary zoospores release motile, biflagellate zoospores which encyst and develop into a germling by growing a germ tube, which eventually develops into a mycelium (Olson *et al.* 1984, Willoughby *et al.* 1995).

1.2.3 Ecology

Knowledge of the ecology of *A. invadans* is limited as it has never been identified or isolated other than from EUS-infected fish. While it is often associated with poor quality water (Tonguthai 1985, Lilley *et al.* 1992), it is not clear whether this reflects the distribution of *A. invadans* itself or merely that of susceptible hosts.

Studies on cultured *A. invadans* have shown growth at salinity of up to 8‰ (Fraser *et al.* 1992), though the upper salinity limit for sporulation may be much lower.

Willoughby (1993) found that the optimal temperature for mycelial growth in culture is around 30°C, which is less than that of saprophytic *Aphanomyces* spp. from the same geographical region. On the other hand, outbreaks are rarely recorded when temperatures are that high (Tonguthai 1985) and *A. invadans* cannot infect even the highly susceptible snakehead at that temperature (Chinabut *et al.* 1995). Willoughby's (1993) study also showed that *A. invadans* died at 37°C in culture, which is less than the highest seasonal temperatures regularly recorded across much of its range, yet EUS still recurs in the same places on successive cold seasons (Tonguthai 1985, Chinabut 1998).

It is unlikely that any of these ambiguities will be resolved until the biology of *A. invadans* outside its hosts has been elucidated.

1.3 Host interactions

Although many studies have identified and described the presence of *A. invadans* in EUS-infected fish, relatively few have considered the mechanisms by which it enters the fish and suppresses or overcomes its immune responses.

This section reviews the published studies of the way in which *A. invadans* interacts with host species, and also with species that are resistant to EUS.

1.3.1 Infection of susceptible fish

A large scale study in India found that the pathology of EUS was remarkably similar in different species (Viswanath *et al.* 1997, Vishwanath *et al.* 1997, 1998). The first clinical signs are usually small, pinhead sized skin lesions, with very little apparent disruption around them. Histopathological examination at this stage reveals hyphae associated with necrosis and inflammation (Vishwanath *et al.* 1997). The size of the lesions then grows, as does the extent of the necrosis. Granulomata usually form around the hyphae while the lesions are 2-4cm in diameter. If unchecked, the lesions continue to expand in width and depth, exposing underlying musculature and skeleton while necrosis, inflammation and granulation replace much of the muscle tissue (Vishwanath *et al.* 1997).

Vishwanath *et al.*'s (1997) description was very similar to those of studies on ornamental dwarf gourami *Colisa lalia* in Japan (Wada *et al.* 1994) and mullet in Australia (McKenzie & Hall 1976), and Lilley *et al.* (1998) regarded it as a standard course of infection.

It is not clear what the final cause of death is, but the levels of blood haemoglobin and serum protein drop severely during the course of infection (Cruz-Lacierda & Shariff 1994) suggesting that potentially fatal serodilution may take place through open lesions. Alternatively, opportunistic bacterial infections develop rapidly in the open lesions, which may be the ultimate cause of death (Boonyaratpalin 1989).

The diversity of oomycete saprophytes that may infect damaged tissue but do not act as primary pathogens (Srivastava 1980) suggested that *A. invadans* must express powerful virulence factors, but very little has been done to establish what they are. *A. invadans* does have haemagglutinating and haemolytic properties (Kurata *et al.* 2000a), though what role they play in infection is not known.

1.3.2 Acquired immunity

Fish may respond to pathogens by producing antibodies specific to those pathogens in a similar manner to higher vertebrates (Acton *et al.* 1971). However, most of the studies that have examined the antibody response of fish to hyphal pathogens have been on the response to *A. invadans*. One exception found anti-*Saprolegnia* antibodies in Atlantic salmon exposed to saprolegniasis (Section 1.4.2), but did not consider fish that had never been exposed so it was unclear whether the antibodies were actually a response to the pathogen or were non-specific (Hodkinson & Hunter 1970).

Antibodies that react to *A. invadans* and saprophytic *Aphanomyces* spp. have been found in striped snakeheads from an EUS-endemic region (Thompson *et al.* 1997) and EUS-naïve rainbow trout *Oncorhynchus mykiss* (Thompson *et al.* 1999). Both species developed specific antibodies infected by *A. invadans*.

It was not established whether the antibody response afforded protection in either case.

1.3.3 Studies on resistant species

A few studies have focused on species resistant to EUS to try to establish the basis of their resistance. Wada *et al.* (1996) injected *A. invadans* into resistant common carp and susceptible ayu, and compared the progressive histopathology at the injection site. While the response of ayu was similar to that described above (Section 1.3.1), carp produced granulomata much more rapidly, containing and destroying the fungus before it progressed beyond the injection site. They also observed that the carp granulocytes fused to form multinucleate giant cells (MGCs) (Secombes 1985) much earlier than in ayu.

Khan *et al.* (1998) conducted a similar study, and found that tilapia showed very little response to injection that was apparent by histopathology, though a few showed granulation and MGC formation.

1.4 Oomycoses of Aquatic Animals Similar to EUS

Very few oomycetes cause disease to animals although there are several which may function as secondary infections on moribund tissue. However, there are three species of oomycete that are known to be highly pathogenic to aquatic animals, including one other *Aphanomyces* sp. and two *Saprolegnia* spp. Comparison of EUS with similar diseases may be valuable, as they are all from the same family and may share characteristics. Also, the other diseases have been studied with approaches that have not been applied to the study of EUS, especially with regard to host interactions.

This section presents a brief review of research on other important oomycoses, with particular emphasis on studies of host interactions.

1.4.1 Opportunistic oomycoses

Neish & Hughes (1980) listed ten genera of oomycetes that contain species pathogenic to aquatic organisms, including such ubiquitous examples as *Achlya* and *Leptolegnia* (Section 1.2.1). If they were all obligate pathogens, then regular outbreaks caused by oomycetes would be expected. In fact, large scale oomycoses are rare and usually attributable to one of a few species such as *A. invadans* or *Saprolegnia parasitica* (Section 1.4.2), although oomycoses among wild fish may be overlooked as studies tend to focus on the relatively few species that are economically important (Shah *et al.* 1977).

Part of the explanation was provided by Srivastava's (1980) finding that oomycetes that normally occur in the same ponds as healthy fish may induce pathology if injected into the fish. Apparently, the oomycetes in question were unable to infect the fish under natural conditions, but once the external defences of the fish were bypassed, they were able to proliferate. Such species are not true pathogens as they cannot infect healthy hosts, but their behaviour is not purely saprophytic either, as they grow on living organisms and contribute to the pathology of those organisms. Hughes (1994) categorised organisms that grow on moribund tissue on living organisms as 'perthotrophs', though most of the oomycetes that fall into that category also behave as saprophytes.

Fish may be damaged naturally, or by handling in the case of farmed fish, which may allow such opportunists a mechanism of infection. For example, handling of sockeye salmon *Oncorhynchus nerka* during administration of antibiotics rendered them prone to such infections (Strasline & McBride 1979). Similarly, many such oomycetes have been isolated from lesions of fish infected with EUS (Roberts *et al.*

1993) and UM (Dykstra *et al.* 1986), though none showed any ability to infect fish on their own.

The presence of such opportunists may confuse efforts to find primary pathogens in diseases of unknown aetiology. It may be speculated that they contribute to the pathology of lesions by preventing them from healing, increasing serodilution and allowing the entry of still more secondary infections, though no study has been done to establish this.

1.4.2 Saprolegniasis

Saprolegniasis has the distinction of being the first fungal disease ever recorded in a vertebrate (Arderon 1748). It returned to prominence in 1877, when an outbreak was reported among salmonids on the border of England and Scotland. The outbreak spread during subsequent years, causing sufficient concern to warrant a parliamentary enquiry, and a white mycelium on the body and fins of the infected fish was classified as *Saprolegnia ferax*. There was some doubt as to whether it was an opportunist taking advantage of lesions caused by an unknown primary pathogen or whether the mycelium was the primary pathogen itself (Buckland *et al.* 1880, as cited in Hughes 1994). Such debate in the first record of a fungal epizootic is of particular interest, as no discussion of fish mycoses seems to be complete without the same point being argued.

Of course, many opportunists were present (Section 1.4.1), but eventually it was established that the common factor in all outbreaks was a *Saprolegnia* sp., which was found up to 1.5cm deep in muscle tissue, much deeper than typical opportunists (Stuart & Fuller 1968, Willoughby 1968). However, the classification of the apparent pathogen has been a cause of considerable confusion since Buckland *et al.* (1880, as cited in Hughes 1994) first described it. Saprolegniasis outbreaks in North America were attributed to an oomycete classified as *S. parasitica* (Coker 1923, as cited in Hughes

1994) that, like *A. invadans*, did not have a sexual stage. However, later studies reported sexual stages, though it was not clear whether they were actually studying the same species (Kanouse 1932, as cited in Hughes 1994). Taxonomic inconsistencies accumulated to the extent that ‘general confusions . . . still prevail after nearly 250 years’ (Hughes 1994).

The observation that the hairs on the cysts of pathogenic isolates are visibly longer than those of saprophytes by microscopic examination (Hatai & Hoshiai 1993, 1994) finally suggested a way of identifying the different species. The application of molecular techniques allowed the various asexual isolates from infected fish to be further divided into the pathogenic *S. parasitica* and the essentially saprophytic *S. ferax* and *S. diclina* (Beakes *et al.* 1994).

Immersion challenges have not always been able to induce saprolegniasis in healthy fish, even after heavy abrasion (Howe & Stehly 1998), although Tiffney (1939) found that wounding increased the susceptibility of several species. Saprolegniasis was induced in coho salmon *Oncorhynchus kisutch* by abrasion after starvation (Hatai & Hoshiai 1993), in temperature stressed and abraded rainbow trout (Howe & Stehly 1998) and in rainbow trout stressed by cortisol implants or injection (Pottinger & Day 1999). The need for stress factors suggests that *S. parasitica* is essentially opportunistic, though *S. diclina* was unable to infect coho salmon treated in the same way as those infected with *S. parasitica* (Hatai & Hoshiai 1993), indicating that some sort of virulence must be present in the latter. Willoughby *et al.* (1983) showed that zoospores are attracted to fish extracts, and germinate much faster in their presence.

Although most studies on saprolegniasis have focused on salmonids, a wide range of fish and amphibians (Tiffney 1939, Kiesecker & Blaustein 1997) are susceptible. However, Tiffney (1939) found that some species such as the black crappie

Pomoxis nigromaculatus (= *P. sparoides*) and Greek chub *Semotilus atromaculatus* are only susceptible after wounding and one species, the American eel *Anguilla rostrata* (= *A. chrysypa*), is completely resistant.

Studies on brown trout *Salmo trutta* have shown that susceptibility to saprolegniasis is affected by a number of factors. Hatchery reared brown trout are more susceptible than wild fish (Richards & Pickering 1978). Mature males are more susceptible than mature females, and both are more susceptible than immature fish (Pickering & Christie 1980). Other studies on Arctic char *Salvelinus alpinus* showed considerable variation in susceptibility between family groups, indicating a significant genetic component to resistance variability between species (Nilsson 1992).

The first signs of infection are grey white areas on the skin, which develop haemorrhagic ulcers and visible fungal growth (Stuart & Fuller 1968). Death may be caused by haemodilution, osmoregulatory failure (Richards & Pickering 1979) or asphyxiation by obstruction of the pharynx (Bruno & Stamps 1987).

The immune response of the fish is not well known, and is probably not very consistent if the disease can only occur in fish that are already highly stressed. Xu & Rogers (1991) found no apparent cellular response in channel catfish *Ictalurus punctatus* infected with *S. parasitica*. On the other hand, López-Dóriga & Martínez (1998) found non-leukocytic ‘capsule cells’ surrounding the hyphae, though they did not appear to afford much protection and it was not clear whether they were interacting with the hyphae at all. The concentration of circulating leukocytes was reduced in brown trout (Álvarez *et al.* 1988), though whether that is a result of an immune response or haematopoietic failure was unclear. Hodkinson & Hunter (1970) found non-specific antibodies to an unspiciated *Saprolegnia* in Atlantic salmon, though it was not established whether they were protective. Brown trout mucus reduces the growth and

branching of the mycelium (Wood *et al.* 1988), though evidently it does not provide complete protection.

While most studies on saprolegniasis have involved salmonids, it is also a major constraint to hatchery production in Africa (Hecht & Endemann 1998). *S. ferax* is a serious pathogen of amphibian eggs and larvae in North America, which has been implicated in a decline in the populations in some species (Kiesecker & Blaustein 1997, 1999).

1.4.3 Winter saprolegniasis

Losses of channel catfish following abrupt temperature drops have commonly been reported in the southern USA. Bly *et al.* (1992) isolated a *Saprolegnia* sp. from such an outbreak, but found no other pathogens. They concluded that their isolate was the primary pathogen of the disease they termed winter saprolegniasis.

Subsequent studies showed that winter saprolegniasis was only contracted at high zoospore concentrations immediately after a cold snap (Bly *et al.* 1993b). The sudden drop in temperature immunosuppressed the fish, making them vulnerable, but they quickly adapted and ceased to be vulnerable regardless of the number of zoospores present (Bly *et al.* 1993b, Howe *et al.* 1998).

A number of immunosuppressive mechanisms of cold shock have been identified, though it is not clear which are directly relevant to winter saprolegniasis. One of the most likely is the large scale loss of mucus-producing cells, which is soon reversed when the fish acclimatise to the lower temperature (Xu & Rogers 1991, Quiniou *et al.* 1998). An almost complete lack of leukocytic infiltration around the site of infection is characteristic of natural outbreaks, and may be a symptom of temperature mediated suppression (Bly & Clem 1992). The concentration of the acute phase protein phosphorylcholine-reactive protein (PRP) is heavily reduced by cold shock, though it is

unclear whether it has any function in defence against winter saprolegniasis (Szalai *et al.* 1994).

Winter saprolegniasis may be controlled with formalin or the herbicide diquat (Bly *et al.* 1996). Studies *in vitro* indicated that the bacterium *Pseudomonas fluorescens* inhibits mycelial growth, though it has not been assessed as a control measure in the natural situation and may cause disease of fish in itself (Bly *et al.* 1997).

1.4.4 Crayfish plague

Crayfish plague or krebspest was first reported in Italy in 1865 (Ninni 1865, as cited in Alderman *et al.* 1984). From there it spread across Europe, extending its range from France to Finland to Russia by the beginning of the twentieth century (Alderman 1996). Its current range encompasses most of Europe, as it reached its current limits in Britain and Ireland in the west, Norway in the north (Alderman 1996) and Turkey in the south east (Rahe & Soylu 1989) in the 1980's.

The aetiological agent was recognised as an oomycete fairly early in the study of the disease (Leuckart 1884, as cited in Alderman *et al.* 1984), and later described as the oomycete *Aphanomyces astaci*. A typical infection involves penetration of the cuticle, principally with extracellular proteases and esterases (Söderhäll & Unestam 1975). Once the tissues are penetrated, the lesions become melanotic within a few hours (Unestam & Weiss 1970). As the disease progresses, the abdominal musculature becomes whitened, limb movements become aphasic, tail-flick response is weakened and the crayfish become so apathetic that wild crayfish are often found in the open in daylight (Alderman *et al.* 1984).

The extreme virulence of *A. astaci* was illustrated when Alderman *et al.* (1987) caused 100% mortality among white-clawed crayfish *Austropotamobius pallipes* by immersion challenge with only 5 zoospores/ml⁻¹. Due to the continuous production of

zoospores by zoosporangia (Rahe & Soylu 1989), mass mortalities are common (Alderman *et al.* 1984, Rahe & Soylu 1989).

A. astaci zoospores are attracted to wounds or areas of weakened cuticle (Nyhlén & Unestam 1980), but are capable of penetrating healthy intersegmental cuticle by a combination of enzymatic and mechanical action (Nyhlén & Unestam 1975). Unestam & Weiss (1970) showed that infected crayfish respond to infection by rapid accumulation of haemocytes and melanisation, and that melanisation occurs far more rapidly in the resistant signal crayfish *Pacifastacus leniusculus* than the susceptible noble crayfish *Astacus astacus*.

In order to revive the European crayfish culture industry, resistant North American crayfish species were introduced into many regions. However, it has since been discovered that some of these species, such as red swamp crayfish *Procambrus clarkii* (Diéguez-Urbeondo & Söderhäll 1993) and signal crayfish (Unestam & Weiss 1970) carry *A. astaci* as a chronic pathogen. In fact, crayfish plague was not found in Britain at all until North American species were introduced, and signal crayfish have been consistently found at outbreak sites (Alderman 1993). In one case, native white-clawed crayfish *Austropotamobilius pallipes* in a closed system were infected, and the only possible source of the infection was a group of signal crayfish in the same system (Alderman *et al.* 1990).

Huang *et al.* (1994) used RAPD analysis to show that there are two distinct genetic groups within the *A. astaci* population of Europe. They surmised that one was responsible for the initial outbreaks in the nineteenth century and that the second was introduced with the north American species in the late twentieth century. Further RAPD analysis supported such a suggestion by revealing that isolates of *A. astaci* in Britain,

which appeared soon after the first introduction of American species, belong to the group that was recently introduced (Lilley *et al.* 1997a).

Crayfish plague is one of the few oomycoses to have a major ecological impact. Its importance stems partly from its high virulence, which enables it to cause massive mortalities among otherwise healthy wild crayfish, and partly from the ability of introduced species to act as vectors without contracting serious symptoms themselves (Alderman 1993). Although there is some evidence that crayfish may be immunised against *A. astaci* (Unestam & Weiss 1970), most studies have shown that exposure to *A. astaci* leads to such high mortalities that there are unlikely to be many survivors to benefit from any acquired immunity (Matthews & Reynolds 1990). Any survivors of a wild outbreak in Europe are now likely to have to face competition from introduced resistant species (Holdich & Domaniewski 1995), which may be a factor in the complete exclusion of European crayfish from many areas.

While programs of reintroduction of white-clawed crayfish have had some success in Britain (Alderman 1993), the continued presence of signal crayfish and red swamp crayfish in European waters make it unlikely that native populations will ever fully recover.

1.5 Aims of Present Project

The objectives of the present study were to assess methods that may be used for the monitoring and control of EUS.

Currently, the main method for monitoring EUS is by histopathological examination of apparently infected fish (Section 1.1.5), which has two major drawbacks. The first drawback is that it is not specific for *A. invadans*, so an experienced pathologist is needed to identify the disease by histopathological

characteristics (Section 1.3.1). The second is that it can only be identified after apparently infected fish are identified and sent to a diagnostic laboratory. In practice, this usually only occurs when a major outbreak is already in progress, so it is too late to take action to mitigate the worst impacts of the outbreak (Section 1.1.3). A further disadvantage of relying on fishkills to mark the presence of EUS is that there is evidence that it may be present without causing them (Section 1.1.4), so it may be overlooked.

The intention of the present study were to assess the possibility of an immunohistochemical diagnostic test using a monoclonal antibody, which would be specific for *A. invadans*. Also, an alternative method of monitoring disease using the presence of antibodies in EUS-exposed fish (Section 1.3.2) was considered, as such a method would allow the identification of a risk from EUS without the need for mass mortalities to mark its presence. Improved monitoring of EUS by either method may enable farmers to take precautions (Section 1.1.6) before they start suffering losses caused by EUS.

Studies of methods of control were aimed at enhancing the immune response of susceptible fish to reduce the impacts of infection by stimulation of the immune system. Before potential immunostimulants could be identified, the interaction between the immune system of host species and *A. invadans* was examined to identify the immune mechanisms that may respond to stimulation.

In a wider context, it was intended that knowledge and techniques would be developed that would facilitate the monitoring and control of EUS, to the benefit of the many aquaculturists whose livelihoods are threatened by it.

2 General Materials and Methods: Culture, Detection and Identification of *Aphanomyces invadans*

2.1 Culture of Oomycetes

2.1.1 Media

Media were prepared after Lilley (1997) in distilled water, and sterilised by autoclaving at 121°C at 2 bars for 15min. All reagents were acquired from BDH Laboratory Supplies (Poole, UK) unless otherwise specified.

Peptone-glucose media one (PG-1) contained 16.65mM glucose, 6g l⁻¹ mycological peptone (Oxoid, Basingstoke, UK), 12 g l⁻¹ technical agar no. 3 (Oxoid), 0.84mM MgCl₂.6H₂O, 1.02mM CaCl₂.2H₂O, 4.96mMKCl, 96.48μM FeCl₃.6H₂O and 0.044g l⁻¹ di-sodium ethylenediaminetetraacetic acid (EDTA). The glucose was dissolved in sodium phosphate buffer, which was prepared by mixing 40.75% v/v solution A (0.20M NaH₂PO₄.2H₂O) with 9.25% v/v solution B (0.40M l⁻¹ Na₂HPO₄.12H₂O) and adjusting the pH to 6.3. Other reagents were dissolved in distilled water, and both solutions were made up to half the final volume, autoclaved and mixed to make PG-1.

Glucose peptone yeast (GPY) broth contained 16.7mM glucose, 1g l⁻¹ mycological peptone, 0.5g l⁻¹ yeast extract (Oxoid), 0.52mM MgSO₄.7H₂O, 0.014 0.102mM KH₂PO₄, 0.20mM CaCl₂.2H₂O, 8.88μM FeCl₃.6H₂O, 9.10μM MnCl₂.4H₂O, 0.016mM CuSO₄.5H₂O and 0.014mM ZnSO₄.7H₂O. GPY agar was made by adding 12g l⁻¹ technical agar no. 3 to GPY broth.

Germedia was GPY broth prepared with reagents at double the concentrations described above, supplemented with 100mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to stimulate germination (Deacon & Saxena 1998).

V8 broth was prepared with 5% v/v V8 vegetable juice (Campbell Grocery Products Ltd., King's Lynn, UK) supernatant after centrifuging at 500g for 10min, and 0.20M CaCO_3 . The pH was adjusted to 6.1.

Autoclaved pond water (APW) was prepared with pond water from Loch Airthrey, Stirling, UK or from a body of water adjacent to the National Inland Fisheries Institute (NIFI), Bangkok, Thailand. The water was filtered through Whatman (Whatman International Ltd., Maidstone, UK) 541 filter paper before autoclaving (Willoughby & Roberts 1994).

All solutions are described in Appendix I.

2.1.2 Long Term Maintenance and Culture

Long term storage was carried out by placing fungi in 25ml tubes containing PG-1 agar slopes, immersed in light, colourless paraffin (BDH) (Smith 1991) sterilised by heating at 120°C for 30min or autoclaving. Isolates were kept for up to six months at room temperature, after which they were sub-cultured (Lilley *et al.* 1998).

For sub-culturing of stocks or experimental use, the mycelium was grown on GPY agar at 20-30°C.

2.1.3 Sporulation and Germination

Initially, mycelium was collected by placing autoclaved hemp *Cannabis sativa* seeds on the edges of the mycelium growing on GPY agar (Khan *et al.* 1998). This approach was followed for antigen collection and the initial antigen preparation and screening in Chapter 3 and throughout Chapters 5 and 6. However, it was found that the hemp seeds

increased the risk of bacterial contamination, so the method of excising agar plugs of diameter 3-5mm from the growing edge of the mycelium with a cork borer or scalpel was adopted.

The plugs or seeds were placed in V8 medium where possible. However, difficulties in supply necessitated the use of GPY broth as a substitute for the later screening stages of Chapter 3 and throughout Chapters 4 and 7. The mycelium was grown in broth for 1-2d at 20-30°C after either collection method.

Isolate 3501d was sporulated using hemp seeds and V8 medium, as sporulation could not be induced by any other method (Chapter 3).

Sporulation was induced by washing the plugs or seeds in six petri-dishes filled with autoclaved distilled water and placing in APW overnight at 24-26°C (Khan *et al.* 1998). No more than five plugs or seeds were washed in one sequence of six petri-dishes.

Germlings were collected by diluting the zoospore suspension with an equal volume of germedia and incubating at 24°C for 1d.

Any culture observed to be contaminated was immediately discarded.

2.1.4 Experimental Isolates

Table 2.1 lists the *Aphanomyces invadans* isolates used for this study. It has been reported that the pathogenicity and sporulation of some oomycete pathogens are attenuated by long periods in culture (Lord & Roberts 1986), and these effects were observed with *A. invadans* in the course of the present thesis. Consequently, the most recent isolate of *A. invadans* was used for every study in order to use isolates as similar as possible to naturally occurring *A. invadans*.

Table 2.1. Details of *Aphanomyces invadans* isolates used in the current study.

Isolate	Species	Species isolated from	Date isolated	Location	Isolated by
PA8*	<i>Aphanomyces invadans</i>	Striped snakehead <i>Channa striata</i>	Jan 1995	Nonthaburi, Thailand	Dr. JH Lilley
NJM9701*	<i>Aphanomyces invadans</i>	Ayu <i>Plecoglossus altivelis</i>	Aug 1997	Shiga Prefecture, Japan	Prof. K. Hatai
B99C*	<i>Aphanomyces invadans</i>	Reba <i>Cirrhinus reba</i>	Mar 1999	Mymensingh, Bangladesh	Dr. JH Lilley
T99G2*	<i>Aphanomyces invadans</i>	Giant gourami <i>Osphronemus gouramy</i>	Nov 1999	Bangkok Noi Thailand	Miss. V. Panyawachira
UM3*	<i>Aphanomyces</i> sp.	Menhaden <i>Brevoortia tyrannus</i>	Aug 1998	Wicomoco River, Maryland, USA	Dr. V. Blazer
FDL458	<i>Aphanomyces astaci</i>	White-clawed crayfish <i>Austropotamobius pallipes</i>	Unknown	River Arrow, Hertfordshire, UK	Dr. D.J. Alderman
ASEAN1	<i>Aphanomyces laevis</i>	Fish pond	Jan 1994	Kasetsart, Bangkok, Thailand	Dr. L.G. Willoughby
SA11	<i>Aphanomyces</i> sp.	Striped snakehead <i>Channa striata</i>	Jan 1995	Nonthaburi, Thailand	Dr. J.H. Lilley
84-1240 (=ATCC 62427)	<i>Aphanomyces</i> sp.	Menhaden <i>Brevoortia tyrannus</i>	May 1984	North Carolina, USA	Dr. M.J. Dykstra
99ExtAph	<i>Aphanomyces</i> sp.	Striped snakehead <i>Channa striata</i>	Mar 1999	AAHRI wet lab, Thailand	Dr. J.H. Lilley
WSA	<i>Aphanomyces</i> sp.	Fish pond water	Sep 1994	Suphanburi, Thailand	W. Valairatana

Table 2.1. Details of *Aphanomyces invadans* isolates used in the current study.

Isolate	Species	Species isolated from	Date isolated	Location	Isolated by
ACHLYA99	<i>Achlya</i> sp.	Climbing perch <i>Anabas testudineus</i>	Dec 1999	Outside fish tank, AAHRI, Thailand	Dr. J.H. Lilley
P32	<i>Saprolegnia ferax</i>	Lake water	1957	Lake Windermere, UK	Dr. L.G. Willoughby
795 (=ATCC 42060)	<i>Saprolegnia australis</i>	Skelly <i>Coregonus lavaretus</i>	1977	Ullswater, UK	Dr. L.G. Willoughby
TP41 (=ATCC 42062)	<i>Saprolegnia parasitica</i>	Brown trout <i>Salmo trutta</i> L.	1970	Hatchery, Windermere, UK	Dr. L.G. Willoughby
E3 (=ATCC 36144)	<i>Saprolegnia diclina</i>	Lake water	1960	Lake Windermere, UK	Dr. L.G. Willoughby
TF20S	<i>Saprolegnia</i> sp.	Striped snakehead <i>Channa striata</i>	Dec 1991	Udon Thani, Thailand	Dr. L.G. Willoughby
TF23	<i>Saprolegnia</i> sp.	Striped snakehead <i>Channa striata</i>	Dec 1991	Udon Thani, Thailand	Dr. L.G. Willoughby
TF29	<i>Saprolegnia</i> sp.	Swamp eel <i>Monopterus alba</i>	Dec 1991	Sakhon Nakhon, Thailand	Dr. L.G. Willoughby
3501d	<i>Leptolegnia caudata</i>	Unknown	1980	India	Prof. M.W. Dick

* isolates shown to be invasive in striped snakehead by injection challenge.

2.2 Identification of *A. invadans* in Infected Fish

2.2.1 Overview

Histopathology was selected as the most appropriate method for diagnosing EUS in this thesis (Section 1.1.5). As all samples in the course of this thesis were taken from artificially infected fish kept in tanks, it was extremely unlikely that a case diagnosis of EUS would result from anything but the presence of *A. invadans*. On the other hand, the importance of accurate diagnosis in the trials did not permit the inevitable false negatives that would result from diagnosis by isolation.

The best histological stain available at present is Grocott's (1955) methenamine silver stain (GMS), which stains muco-polysaccharides but is not specific to *A. invadans*. Consequently, there is a risk that a pathogen with similar characteristics to *A. invadans* may be misidentified as *A. invadans*, but no such pathogen has ever been reported and GMS is generally considered conclusive (Wada *et al.* 1994, Chinabut & Roberts 1999).

2.2.2 Fixation and Processing

All concentrations of solvents are given as percentage volume in distilled water.

All samples were prepared by fixing in 10% neutral buffered formalin (6.66M formaldehyde [Sigma, Poole, UK], 25.64mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ [BDH], 33.71mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ [BDH]) for at least 24h. Where possible, the formalin was chilled to 4°C before use. Samples were placed in processing cassettes and washed in running water for 10min.

They were then automatically processed (Table 2.2) and embedded in paraffin wax. Sections of 5µm were cut with a microtome and dried on to slides by heating at 40°C overnight.

All staining procedures began with deparaffinisation by 2min immersions in two changes of xylene (Fisher Scientific UK Ltd., Loughborough, UK) and two changes of absolute ethanol (Fisher), followed by 95% ethanol and 70% ethanol. The sections were then washed in running tap water for 5min.

All solutions are described in Appendix I.

Table 2.2. Automatic processing schedule used for preparing sections for histopathology.

Stage	Solution	Time	Stage	Solution	Time
1	50% ethanol	1h	7	Absolute ethanol	2h
2	80% ethanol	2h	8	Chloroform	1h
3	8% phenol meths	2h	9	Chloroform	2h
4	8% phenol meths	2h	10	Paraffin wax	2h
5	8% phenol meths	2h	11	Paraffin wax	3h
6	Absolute ethanol	2h	12	Paraffin wax	3h

2.2.3 Grocott's Methenamine Silver Stain (GMS)

All solutions were prepared in distilled water, and all reagents were acquired from Sigma.

After deparaffinisation, slides were oxidised in 5% chromic acid (0.50M CrO₃) for 1h and washed in running water for 10min. They were treated in 96.1mM sodium bisulphite for 2min, washed in running water for 5min and washed in three 2min changes of distilled water.

Silver nitrate working solution was prepared immediately before use with 50% v/v methenamine silver nitrate solution (0.21M methenamine, 14.74mM AgNO₃) and 4% v/v borax solution (0.13M Na₂B₂O₇·10H₂O). Slides were placed in silver nitrate

working solution preheated to 58°C in a water bath and incubated until sections appeared light brown, which took 10-20min. They were washed in three further 2min changes of distilled water, toned in 2.54mM gold chloride for 2min, washed again with three 2min changes of distilled water, treated in 0.13M sodium thiosulphate for 2 min and washed in running water for 5min (Grocott 1955).

One of the following counter stains was then applied.

All solutions are described in Appendix I.

2.2.4 Haematoxylin and Eosin Stain (H&E)

H&E (Drury & Wallington 1980) alone may be used to detect EUS as it shows the characteristic granulomata. The hyphae themselves are also visible in advanced infections, though they are far less clear than when stained by GMS. Alternatively, GMS-H&E staining uses H&E as a counter stain to GMS (Wada *et al.* 1994), which combines the clear staining of hyphae of GMS with the histopathological detail provided by H&E.

All solutions for H&E were prepared in distilled water unless otherwise specified, and all reagents were acquired from BDH unless otherwise specified.

After deparaffinisation for H&E alone, or after the application of sodium thiosulphate in GMS, the slides were stained in Mayer's haematoxylin (3.31mM haematoxylin, 1.01mM NaIO₃ [Sigma], 0.20M AlK(SO₄)₂.12H₂O, 5.20mM citric acid, 0.30M C₂H₃Cl₃O₂) for 15min. They were washed in running water for 2min, given three quick dips in acid alcohol (2.74M HCl in methylated spirits [Fisher]), washed again, placed in Scott's tap water substitute (41.66mM NaHCO₃, 81.14mM MgSO₄.7H₂O in tap water) for 1min and washed again.

Eosin was prepared with 89% v/v 1% eosin solution (10g l⁻¹ eosin yellowish) and 11% v/v Putt's eosin (14.45mM eosin yellowish, 17.0mM K₂Cr₂O₇, 10% v/v

saturated aqueous picric acid, 10% v/v ethanol [Fisher]). The slides were placed in eosin for 5min and washed in tap water for 5min. They were then dehydrated by 5min each in methylated spirits (Fisher), two changes of absolute ethanol (Fisher) and xylene (Fisher). They were placed in a final change of xylene (Fisher) and cover slips were attached with pertex (Cellpath PLC., Hemel Hempstead, UK).

2.2.5 Rapi-diff Staining

Rapi-diff (Raymond A. Lamb laboratory supplies, London, UK) is a commercially available kit designed for staining cell cultures which can be adapted for use with tissue sections. After washing, excess water was removed and the slides were stained in eosin Y for 2min. Excess eosin was removed and the slides were dipped in methylene blue, then washed in running tap water for 10min.

Slides were allowed to air dry before cover slips were applied as described above.

2.2.6 Microscopy and Photography

Slides were viewed using an Olympus (Southend-on-Sea, UK) BX40 microscope. For photography, an Olympus SC35 camera with 64ISO 35mm slide film was attached. Shutter speeds were determined automatically.

3 Production and Characterisation of Monoclonal Antibodies to *Aphanomyces invadans*

3.1 Introduction

The specific immune system of mammals is frequently utilised by microbiologists to produce antibodies to microbes of interest. The antibodies can then be used for a wide range of techniques for characterisation of the study organism, or identification of the organism in histological preparations (Harlow & Lane 1988).

Several oomycetes, including *A. invadans*, have been studied with the aid of polyclonal antibodies (PABs), collected from the serum of rabbits immunised with mycelium or extracellular products (ECPs). Although the antibodies showed distinct differences between electrophoretically separated antigens present in *A. invadans* and saprophytic *Aphanomyces* spp, they recognised some antigens in every saprolegniacean they were tested against, and so were not genuinely specific (Lilley *et al.* 1997c).

Lack of specificity is a commonly reported difficulty in the use of PABs in the study of oomycetes. Murine PABs to *Saprolegnia parasitica* mycelium not only cross-reacted with many other oomycetes, but also with several non-oomycete fungi (Bullis *et al.* 1990). Petersen *et al.* (1996) raised rabbit PABs to *A. eutiches* and improved their specificity by reacting them with other oomycetes before *A. eutiches* itself, but they were still unable to distinguish between different *Aphanomyces* spp.

An alternative approach to antibody studies emerged from the Nobel-prize winning discovery that the antibody-producing B-cells of mice can be fused with cancerous myeloma cells and grown in culture (Kohler & Milstein 1975). The resulting hybridoma cells can be separated into single clones that produce monoclonal antibodies

(MAbs), to a single antigen, which are less likely to cross-react with other species than PAbs, which contain every antibody generated in the immune response. A further advantage is that the hybridoma cell lines are self-replicating and produce the same antibody continuously, while experiments with PAbs are usually unrepeatable as the quantities produced are limited, and even closely related animals may produce different antibody responses to the same antigen (Milstein 1986).

The first MAbs raised to an oomycete were to a crop pest, *Phytophthora cinnamomi*, and identified hitherto unknown species and genus specific antigens (Hardham *et al.* 1985). Since then, MAbs have been used in the study of the life-cycle (Hardham & Suzaki 1986, Estrada-Garcia *et al.* 1989, Estrada-Garcia *et al.* 1990), and taxonomy of oomycetes (Hardham *et al.* 1986, Beakes *et al.* 1995). More practical applications have included the development of a 'dipstick' immunoassay to detect *P. cinnamomi* in soil (Cahill & Hardham 1994) and an enzyme linked immunosorbent assay (ELISA) to detect *Phytophthora* spp. and *Pythium* spp. in water samples (Ali-Shtayeh *et al.* 1991).

Reports of the production of MAbs to oomycetes have described considerable variation in specificity. Bullis *et al.* (1996) found that MAbs to *S. parasitica* cross-reacted with other oomycetes, basidiomycetes and ascomycetes while MAbs produced by Arashima *et al.* (1994) to *Pythium* sp. were apparently specific to the population the antigen was drawn from, though species relationships were unclear in the latter case.

Lilley *et al.*'s (1997c) experiences with PAbs suggested that *A. invadans* has some immunogenic antigens that are not shared with other *Aphanomyces* spp., although the recognition of other antigens rendered their PAb non-specific. MAbs to the species-specific antigens would provide a means of distinguishing *A. invadans* from other oomycetes. Also, the fact that such antigens were not found in saprophytes suggests that

they may be involved in the pathogenicity of *A. invadans*. In the present study, MAbs were raised to the germlings of *A. invadans*. These MAbs were characterised with respect to the antigens that they recognise prior to using them for diagnosis and studies of the pathology of EUS.

3.2 Methods

3.2.1 Reagents

All reagents were obtained from Sigma (Poole, UK) unless otherwise specified. All reagents were prepared in distilled water, unless otherwise specified.

Phosphate Buffered Saline (PBS) was 5.62mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (BDH), 14.4mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (BDH) and 0.15M NaCl at pH 7.2. The fixative solution for antigen preparation was prepared by dissolving 0.49M formaldehyde in PIPES buffer (50mM Piperazine-N,N'-bis[2-ethanesulphonic acid]; 1,4-Piperazinediethanesulphonic acid [PIPES], pH 6.8) (Burr & Beakes 1994). Glycine solution was 75mM glycine in PIPES buffer.

Hybridoma maintenance medium was Dulbecco's Modification of Eagles Medium (DMEM) containing 20mM L-glutamine, 1000U ml^{-1} penicillin, 1mg ml^{-1} streptomycin, 0.5mM sodium pyruvate and 20% v/v foetal bovine serum (FBS) heat inactivated at 55°C for 30min. All references to DMEM include additives unless otherwise specified. Hypoxanthine aminopterin thymidine media supplement (HAT) was prepared as a 50x stock solution in DMEM containing 5mM hypoxanthine, 0.002mM aminopterin and 0.08mM thymidine.

For dot blot analysis, Tris buffered saline (TBS) was 20mM Trizma base and 0.50M NaCl at pH 7.5. Tween supplemented Tris Buffered Saline (TTBS) was prepared by adding 0.1% v/v tween-20 to TBS. Immunoblot developing solution was prepared

immediately before use by adding 2ml chloro-naphthol solution (20.81mM 4-chloro-1-naphthol [BDH] in methanol [Fisher]) and 10 μ l 30% w/w hydrogen peroxide to PBS.

For ELISA, coating solution was 0.001% w/v poly-L-lysine in carbonate-bicarbonate buffer (15mM Na₂CO₃, 23mM NaHCO₃, pH 9.6), and was prepared immediately before use. Glutaraldehyde solution was 0.05% v/v glutaraldehyde in PBS. ELISA blocking solution was 3% w/v casein in PBS. Antibody buffer was 1% bovine serum albumin (BSA) in PBS. Chromogen solution was prepared immediately before use by dissolving 1% v/v of 42mM 3'3'5'5'-Tetramethylbenzidine dihydrochloride (TMB) in a 1:2 solution of glacial acetic acid (BDH): distilled water, and 0.033% v/v 30%w/w hydrogen peroxide in substrate buffer (0.11M citric acid, 100mM sodium acetate, pH 5.4).

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, sample buffer was 25% v/v glycerol, 0.69mM sodium dodecyl sulphate (SDS), 14.4mM 2-mercaptethanol and 1.49mM bromophenol blue (BDH) prepared in 60mM Trizma base at pH 6.8 (Bollag *et al.* 1996). Reservoir buffer was 0.12M Trizma base, 0.96M glycine and 17.34mM SDS at pH 8.3. Transblot buffer was 0.19M glycine and 25.02mM Trizma base prepared in distilled water containing 20% v/v methanol (Fisher) at pH 8.3. Developing solution was prepared in PBS by adding 19.04mM 4-chloro-1-naphthol in methanol at 24% v/v, and 833 μ l l⁻¹ 30% w/w hydrogen peroxide.

All solutions are described in Appendix I.

3.2.2 Preparation of Monoclonal Antibodies

3.2.2.1 Fixation of Germlings

Germlings of *A. invadans* isolate PA8 were prepared (Section 2.1.3) in Petri dishes (Bibby Sterilin Ltd, Stone, UK). Media containing the germlings was collected and centrifuged at 3000g for 20min. The supernatant was removed and the pellet resuspended in fixative solution. After 30min, the fixative solution was centrifuged as before and the supernatant removed. The germlings were resuspended in glycine solution to wash off remaining formaldehyde and centrifuged as before. The supernatant was removed and the pellet was resuspended in PBS (Burr 1991).

If whole germlings were required, they were counted on an improved Neubauer haemocytometer and adjusted to the required concentration with PBS. If soluble antigen was required, germlings were disrupted by shaking with 0.5mm silicon beads in a bead shaker (Biospec Products, Bartlesville, USA) for 160s.

Soluble antigen concentrations in units of mg ml^{-1} were assessed spectrophotometrically by measuring absorbance at 280nm using quartz cuvettes and dividing the absorbance by 1.4 (Bollag *et al.* 1996).

ECPs of 1d germlings were collected (Section 2.1.3). The media was filtered through grade 541 filter paper (Whatman), then through 0.45 and 0.20 μm sterile syringe filters (Sartorius AG, Göttingen, Germany) to remove cellular material. The filtered extract was concentrated approximately 18x by centrifuging for 30min at 3000g in Vivascience concentrators (Sartorius AG) with a 5kDa molecular weight cut-off.

3.2.2.2 Immunisation Schedule

Three BALB/c mice, designated 1g, 2g and 3g, were immunised with 2.8×10^5 whole germlings ml^{-1} in autoclaved PBS mixed 1:1 with the adjuvant Titremax gold (CytRx

Corporation, Norcross, USA) to enhance the B-cell response. Each mouse was injected intraperitoneally with 100 μ l of the mixture. The mice were boosted with 4.0×10^5 germlings ml⁻¹ mixed 1:1 with Titremax 32d later, and a second booster with 2.5×10^5 germlings ml⁻¹ 19d later. The last booster, 28d later was 100 μ l of 2.5×10^5 germlings ml⁻¹ without adjuvant injected into the caudal vein. Fusion was carried out 3d later.

Blood samples were collected before the first injection, and 7-10d after every intraperitoneal injection. Blood was allowed to clot for 2h at room temperature, then serum was collected by centrifuging for 5min at 10,000g and the supernatant was pipetted off. Samples were screened by dot blot (Section 3.2.3.1) to confirm the lack of antibody response before immunisation, and to confirm the subsequent development of the response.

3.2.2.3 Fusion

SP2 myeloma cells were thawed and grown into the mid-log phase in DMEM before the fusion (Section 3.2.2.4).

Each mouse was killed by asphyxiation with carbon dioxide and exsanguinated by cardiac puncture. The spleen was dissected out and placed in DMEM at 37°C. All subsequent procedures were carried out in a laminar flow hood, using sterile instruments, materials and technique.

The spleen was washed in three changes of DMEM without additives. Both ends of the spleen were removed and B-cells were detached by gently passing medium through the spleen with a syringe. The resulting suspension was allowed to stand for 1min and made up to 50ml with DMEM.

SP2 myeloma cells were suspended in 50ml DMEM, and the two suspensions were centrifuged at 150g for 10min. Both pellets were resuspended in 10ml DMEM and

viable cell counts were taken by mixing aliquots of the suspension 1:1 with 0.1% trypan blue (Sigma) and counting unstained cells on an improved Neubauer haemocytometer.

Spleen cells were mixed with SP2 cells at a ratio of 1:10 and centrifuged at 150g for 10min. Some samples of each were kept separately for preparation of controls. The supernatant was removed and the cells were gently resuspended. 1ml of a 1:1 mixture of DMEM and polyethylene glycol (PEG) was added over 1min. The solution was left to stand and swirled occasionally. After 1.5min 1ml DMEM was added over 30s, then 3ml over the subsequent 30s and 16ml over 1min. The suspension was left to stand for 5min, then spun at 150g for 5min. The cells were resuspended in 10ml DMEM and placed in the incubator for 2h.

A blood cell feeder layer was prepared by exsanguinating an unimmunised BALB/c mouse killed by carbon dioxide asphyxiation and placing the blood in 300ml DMEM. The solution was prepared for culture with 2% v/v HAT. The cell suspension was centrifuged at 150g for 10min and resuspended in the medium. Aliquots of 1ml the suspension were placed in the wells of a 24-well culture plate (Iwaki, Japan). Controls of spleen cells and SP2s were prepared to ensure that spleen cells were unable to survive alone, and that SP2s were killed by the aminopterin.

The culture was incubated at 37°C for 10d and screened for positive clones by dot blot analysis.

3.2.2.4 Hybridoma Maintenance and Culture

Protocols for MAb preparation were adapted from Adams *et al.* (1995). All hybridomas used were derived from SP2 myeloma cell lines. General culture was carried out in DMEM in a Galaxy CO₂ incubator (RS Biotech, Finedon, UK) at 37°C in an atmosphere containing 5% carbon dioxide.

For use in assays, hybridomas were cultured until the medium appeared yellow, which usually took 5-15d. The medium was then collected and centrifuged at 1000g for 10min to remove cell debris. The supernatant was collected.

3.2.2.5 Isolation of Monoclonal Cultures

The contents of wells containing supernatant that did not give a positive result in the initial screening (Section 3.2.3) were discarded. The contents of those that did were resuspended in 200µl and placed in a corner well of a 96-well microplate (Greiner Labortechnik). Eight serial doubling dilutions were carried out along the first column of the plate. Serial doubling dilutions were then carried out along each row of the plate.

Distinct clones were visible after 5-10d, when wells containing single clones were marked and screened. In plates where no single clones were positive, wells containing small numbers of clones were screened. Dot blot analysis (Section 3.2.3.1) was used for the first three screenings, after which ELISA (Section 3.2.3.2) was used.

At each stage, positive clones were collected and plated out as previously described. When single clones were plated out to give further single clones testing positive, they were considered monoclonal.

3.2.2.6 Cryopreservation of Hybridomas

Cell lines were cryopreserved in liquid nitrogen for long-term storage. To prepare them for storage, they were collected at high density at the mid-log growth phase and centrifuged at 150g for 7min. The pellet was resuspended in DMEM containing the additives mentioned above and 10% v/v dimethyl sulphoxide (DMSO) at 4°C and placed in a cryopreservation vial (Alpha, Eastleigh, UK). The vial was immediately placed in a cryopreservation chamber filled with iso-propanol (BDH) and incubated overnight at -70°C, then transferred to liquid nitrogen the following day.

To thaw cells from liquid nitrogen, 9ml DMEM without FBS was placed in a centrifuge tube and 1ml inactivated FBS was layered underneath it as a cushion for centrifuging. The cryopreservation vial was removed from liquid nitrogen and placed directly in a water bath at 37°C to thaw the cells. The medium in the vial was layered on to the DMEM without FBS and the tube centrifuged at 150g for 7min. The supernatant was removed and the pellet was resuspended in DMEM which was placed in a 24-well tissue culture plate (Greiner Labortechnik, Stonehouse, UK), and placed in the incubator.

3.2.3 Screening of Hybridoma Supernatants

3.2.3.1 Dot blot

Soluble antigen was prepared as previously described from germling suspensions adjusted to 10^3 germlings ml^{-1} .

The dot blot was carried out using a 96-well bio-dot (Bio-Rad, Hercules, USA). Nitro-cellulose paper (NCP) (BDH) was cut to the appropriate size and shape and pre-wetted in TBS for 20min at 40°C. The bio-dot was assembled with the nitro-cellulose paper between the gasket and the top section, 100 μl TBS was placed in each well and 10 μl of antigen solution was added. The solutions were allowed to drain through the NCP without vacuum. Aliquots of 100 μl TBS were drained through each well, then 300 μl of TBS containing 5% w/v BSA was drained through each well to block non-specific binding sites. Excess protein was washed out with two vacuum assisted washes with 400 μl TTBS and one of 400 μl TBS.

Hybridoma supernatant was placed in duplicate wells in 100 μl aliquots. Positive control wells were prepared with TBS containing 0.01% v/v positive serum collected

from the final exsanguination of the mouse. Negative controls were prepared with TBS only. The wells were washed three times with 400µl TTBS under vacuum.

TBS containing 0.01% v/v horse radish peroxidase (HRP) labelled goat anti-mouse IgG (Diagnostics Scotland, Carlisle, UK) was drained through each well in 100µl aliquots. Three washes of 400µl TTBS and one of 400µl TBS were carried out under vacuum. Development solution was drained through each well in the dark at 100µl per well. The membrane was removed and the reaction stopped by placing in distilled water for 10min. Positive results were indicated by dark dots.

3.2.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

Soluble antigen was prepared (Section 3.2.2.1) and adjusted to a concentration of 1µg ml⁻¹ in PBS.

Hydrophilic Immulon 4 HBX ELISA plates (Dynex Technologies Inc., Chantilly, USA) were polarised by the addition of coating solution for 1h. They were washed twice with low salt wash buffer (LSW), then 100µl antigen solution was added to each well and the plates were incubated overnight at 4°C. After a 20min incubation at room temperature (24°C ± 2) with 50µl glutaraldehyde solution, the antigen solution was removed and the plates were washed three times with LSW. Vacant protein binding sites were blocked with ELISA blocking solution for 2h and the plates washed three times with LSW.

Duplicate wells were loaded with 100µl hybridoma supernatant. Duplicate negative controls were prepared with antibody buffer. Initially, duplicate positive controls were prepared with 0.001% v/v positive mouse serum in antibody buffer, but later assays used hybridoma supernatant previously shown to be positive in order to

conserve mouse serum for other applications. After a 2h incubation, plates were washed five times with TTBS, incubating for 5min on the last wash.

Each well was loaded with 0.001% v/v HRP labelled anti-mouse gamma globulin (Diagnostics Scotland) and incubated for 1h. After a final wash with TTBS as previously described, 100µl chromogen solution was added to each well, which reacted with the HRP to give a blue solution in the case of positive samples. After 10min, 50µl 2M H₂SO₄ was added to each well to stop the reaction and the absorbance of each well assessed in a multiscan spectrophotometer at a wavelength of 450nm. Supernatants that gave an absorbance more than three times that of the mean negative control were considered positive.

3.2.4 Characterisation of Monoclonal Antibodies

3.2.4.1 Isotypes of Monoclonal Antibodies

The isotypes of the MAbs were established by ELISA. Antigen was attached as previously described (Section 3.2.3.2). After washing with LSW, MAb supernatants were applied in eight sets of duplicate wells. After washing with TTBS, 0.001% v/v solutions of goat antibodies specific to the isotypes IgG1, IgG2a, IgG2b, IgG3, IgM or IgA (Sigma) were dissolved in antibody buffer and 100µl of each was applied to duplicate wells for each MAb. Positive controls were prepared with a 0.001% v/v solution of goat anti-mouse gamma globulin in antibody buffer, and negative controls with antibody buffer alone.

After washing with TTBS, 100µl of 0.001% v/v HRP labelled donkey anti-goat IgG (Diagnostics Scotland) in antibody buffer was applied to each well and incubated for 1h. The plates were washed with TTBS and developed (Section 3.2.3.2). Wells containing the appropriate isotype-specific antibody were identified as their absorbance

was over three times that of the negative controls. The assay was carried out twice, ensuring that only one isotype was recognised each time and that the results were replicated.

3.2.4.2 Characterisation of antigens

Denaturing SDS-PAGE (Laemmli 1970, Bollag *et al.* 1996) followed by Western blot was used to establish the molecular weights (MWs) of the antigens recognised by the mouse serum at the time of fusion (Section 3.2.2.3) and the MAbs, and to establish whether the antigens were destroyed by proteinase. All steps were carried out at room temperature (20-25°C) unless otherwise specified.

Soluble germling extract was prepared (Section 3.2.2.1) at a concentration of 1.38mg ml⁻¹. It was not possible to estimate the protein concentration of ECPs due to the presence of protein in the yeast extract of the culture medium.

Some samples were digested with 1mg ml⁻¹ proteinase K (Sigma) at 60°C for 60min. Digested and undigested samples were prepared for electrophoresis by adding sample buffer at 20% v/v, giving a working protein concentration of 1.10mg ml⁻¹ in the undigested germling extract.

Samples and rainbow molecular weight markers with a molecular weight range of 10-250kDa (Amersham Life Sciences, Little Chalfont, UK) were prepared for electrophoresis by boiling for 4min. The samples were centrifuged at 13,000g for 5min. to remove particulate debris.

SDS-PAGE was carried out on a Model SE250 (Pharmacia Biotech, San Francisco, USA) mini-gel system using two 12-lane pre-cast gels with an acrylamide concentration gradient of 4-20% (Sigma) (Lilley *et al.* 1997c). The mini-gel system was filled with reservoir buffer. Eight lanes of each gel were loaded with approximately 40µl of digested or undigested sample each, and a ninth lane in each gel was loaded

with the molecular weight marker. A potential of 100V was applied with a Model 200/2.0 power supply (Bio-Rad) until the bromophenol blue in the sample buffer reached the bottom of the gel.

Western blot was carried out using a TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, USA) for wet blotting. The gels were placed in transblot buffer along with Whatman 1 filter paper and NCP for 10min to equalise their pH. The transphor was assembled with the gels in contact with the NCP and three filter papers on either side. Bubbles were removed from between the papers. The gels were placed on the anode side of the transphor, which was then filled with transblot buffer and 60V was applied for 1h to transfer the samples from the gels to the NCPs.

The NCPs were removed from the transblotter and washed by one quick rinse followed by two 5min incubations in TTBS. Non-specific protein binding sites were then blocked with antibody buffer for 1h. The NCPs were washed again with one quick rinse followed by three 5min incubations in TTBS, and all subsequent washes were carried out in the same way.

The NCPs were cut into strips, each strip corresponding to the sample from one lane of the gel. The strips were placed in mini-incubation trays (Bio-Rad) and incubated with either serum from the mice at the time of fusion diluted 1% v/v in antibody buffer or neat MAb supernatant overnight.

The following day, the strips were washed and incubated with 1% v/v biotin labelled goat anti-mouse IgG (Diagnostics Scotland) in antibody buffer for 1.5h. The strips were washed again and incubated with 1% v/v streptavidin peroxidase (Diagnostics Scotland) in antibody buffer.

After a final wash step, detergent was removed by incubating for 5min in PBS. The blot was developed by incubating in developing solution in the dark until bands appeared in the undigested sample, when all reactions were stopped by sluicing with distilled water and incubation for 10min in distilled water.

The strips were dried and reassembled in the configuration of the original NCPs. The banding patterns were recorded with a Hewlett-Packard (Vancouver, USA) Scanjet ADF scanner.

To calculate the MWs of the antigens recognised, the relative electrophoretic migration (Rf) of each standard was calculated by dividing the distance between the top of the electrophoretic run and the leading edge of the protein band by the total length of the run. The Rf of the markers was regressed cubically against their MWs, giving strong regressions for all samples ($r^2 \geq 0.990$). The Rf of the sample bands were then calculated, and the MWs of those bands calculated using the regression equations.

3.3 Results

3.3.1 Monoclonal Antibodies Raised

All three mice showed an antibody response to *A. invadans* germlings throughout the injection schedule, so fusions were performed on all three, and five MAbs were isolated (Table 3.1). Two were from mouse 1g and designated MAbs 1gAA11 and 1gEE5, one was from mouse 2g and designated MAb 2gMF5 and MAbs 3gFE11 and 3gJC9 were from mouse 3g. All five antibodies were classified as the IgM isotype.

Unfortunately, time constraints prevented the completion of isolation and characterisation of all antibodies detected by the early screenings, and a further 99 cell lines were cryopreserved.

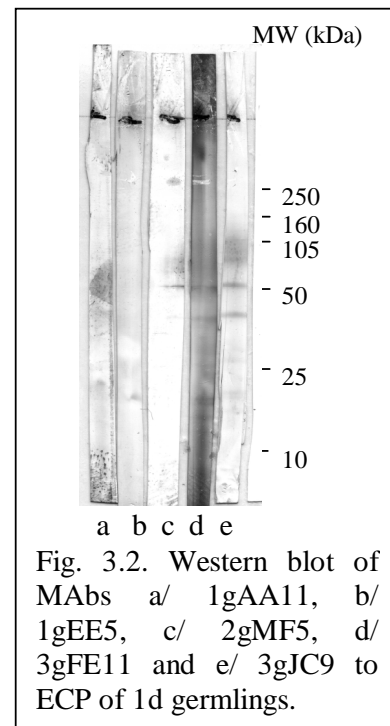
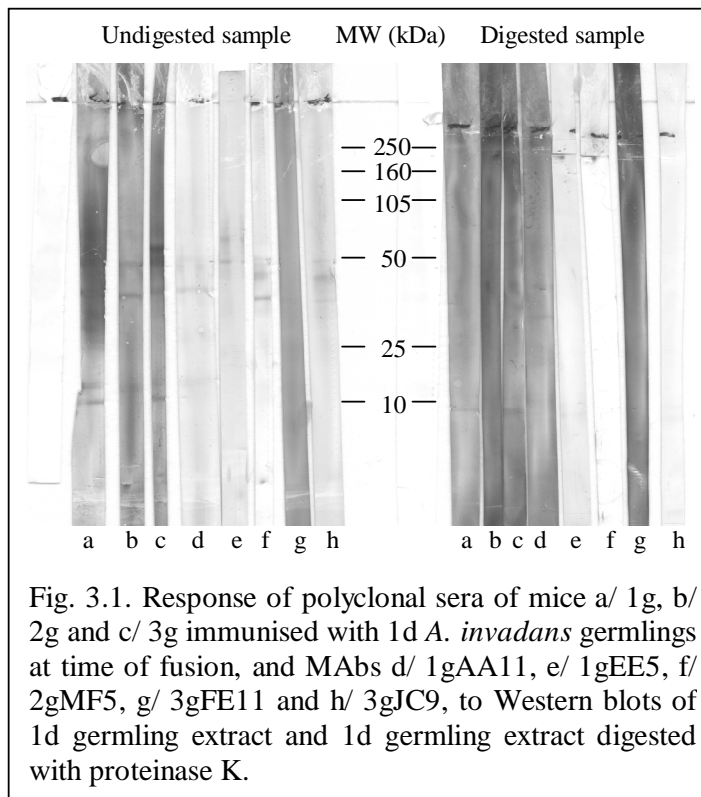
3.3.2 Characterisation of Antigens

The antigens recognised by the polyclonal mouse sera at the time of fusion and the MAbs were characterised by Western blot analysis (Fig 3.1), and the MWs of the bands they recognised were determined (Table 3.1). All MAbs recognised antigens in the soluble extract, but only three recognised antigens in the ECP (Fig. 3.2).

Only one antigen, recognised by MAb 1gAA11, was not removed by proteinase K digestion, demonstrating that all other antigens were proteins in nature.

Table 3.1. Type and MW of antigens recognised by MAbs raised to *A. invadans* germlings.

MAB	MW of antigens (kDa)	Detected after protein digestion	Detected in germling extract	Detected in ECPs
1gAA11	7	No	Yes	No
	34	Yes	Yes	No
	38	Yes	Yes	No
1gEE5	38	No	Yes	No
	50	No	Yes	No
	56	No	Yes	No
2gMF5	33	No	Yes	Yes
	36	No	Yes	No
	38	No	Yes	No
	46	No	Yes	No
3gFE11	23	No	Yes	Yes
	24	No	No	Yes
	25	No	No	Yes
	33	No	No	Yes
	46	No	No	Yes
3gJC9	23	No	No	Yes
	24	No	No	Yes
	25	No	No	Yes
	25	No	No	Yes
	33	No	Yes	Yes
	35	No	Yes	No
	38	No	Yes	No
	46	No	No	Yes
	72	No	No	Yes
	102	No	No	Yes



3.4 Discussion

Five MAbs were raised to *A. invadans* germlings. Three recognised antigens in the ECPs as well as the germlings, while two only recognised the germlings. The mouse PABs recognised several antigens not recognised by any of the MAbs, so there may be more useful antibodies in the MAbs that were not completely characterised.

Enzymes expressed extracellularly are important in the ability of *A. astaci* to infect healthy crayfish (Söderhäll & Unestam 1975, Söderhäll *et al.* 1978). Similarly, the extracellular expression of enzymes has been implicated in saprolegniasis of adult fish (Peduzzi & Bizzozero 1977) and eggs (Rand & Munden 1992).

The presence of ECPs expressed by *A. invadans* is implied by the fact that considerable tissue damage is often found some distance away from any hyphae in EUS-infected fish (McKenzie & Hall 1976, Wada *et al.* 1994, Hatai *et al.* 1994, Vishwanath *et al.* 1998). *A. invadans* mycelium has been shown to have

haemagglutinating and haemolytic properties (Kurata *et al.* 2000a) which are probably involved in pathogenicity, but the question of whether they are expressed extracellularly has not been addressed.

Three of the antibodies in the present study recognised not only ECPs, but also antigens in them that were not found in the germlings themselves. In the case of some of the antigens recognised by MAb 3gJC9, the ECP antigens had lower MWs than those expressed in the germlings. In this case, the increase in number of antigens in the ECPs may be due to large proteins dissociating into smaller units, each containing antibody binding sites, as they leave the germling.

In most other cases, the ECP antigens were as large or larger than the germling antigens so they could not be dissociation products of the antigens expressed in the germling. It is likely that more of these antigens were present extracellularly than in the germlings themselves, though not necessarily in higher density as the culture medium was concentrated (Section 3.2.2.1).

Although MAbs 2gMF5 and 3gFE11 recognised less antigens than 3gJC9, those that they did recognise were of the same MW, implying that the antigen that they reacted with is actually the same molecule. MAbs 2gMF5 and 3gFE11 recognised antigens that gave the strongest bands, so the difference is probably due to 3gJC9 having a higher affinity. It is also possible that the three MAbs recognised different epitopes within a protein that dissociated during electrophoresis, and the epitopes recognised by some of the antibodies were not present on every sub-unit.

It is not clear why one of the 1gAA11 antigens was removed by proteinase K digestion while the other two remained. It is unlikely that the same epitope was present on both a protein and a carbohydrate, so the most likely explanation is that the undigested antigens were also proteins that were protected from digestion in some way.

Low affinities are characteristic of the IgM isotype (Harlow & Lane 1988), and IgM is commonly regarded as being a less useful MAb than IgG (Liddell & Cryer 1991). Nevertheless, around half the useful MAbs reported from other attempts to raise them to oomycetes were IgM (Hardham *et al.* 1986, Estrada-Garcia *et al.* 1989). Although the fact that all of the MAbs raised in the present study are IgM is a cause for concern, it does not preclude the potential of these MAbs as research tools.

3.5 Summary

- Five MAbs were raised to *A. invadans* germlings.
- All of the antibodies recognised antigens in whole germlings, and three recognised antigens that were expressed extracellularly.

4 Optimisation and Assessment of Immunohistochemistry Techniques

Using Monoclonal Antibodies

4.1 Introduction

The specific nature of MAbs has often been exploited by fish pathologists to recognise known fish pathogens within host tissue (Adams *et al.* 1995). Used for immunohistochemistry (IHC), they can be used to confirm or discount the presence of a specific pathogen, which conventional histology techniques may be unable to distinguish from the wide range of opportunists usually present in any fish showing clinical signs of disease. The prevalence of opportunists is a particular problem in the diagnosis of EUS, where the clinical sign is a large skin lesion that invites many diverse secondary infections, confounding any attempt to establish the presence of *A. invadans* (Section 1.1.2).

Most MAbs raised to oomycetes for IHC have been applied to elucidate taxonomic distinctions (Hardham *et al.* 1986, Estrada-Garcia *et al.* 1989, Beakes *et al.* 1995, Cope *et al.* 1996). MAbs raised to *Phytophthora cinnamomi* have also been used to trace the movement of certain antigens during encystment and entry into the host plant (Gubler & Hardham 1988, Cope *et al.* 1996).

Over ten years ago, Bullis *et al.* (1990) suggested that immunoassays ‘may . . . be useful in identifying oomycete infections in clinical cases’ of fish diseases. In spite of the considerable advances in the development of IHC techniques since then (Mayer & Bendayan 2001), only one attempt to use MAbs to locate any oomycete pathogen in host tissues has been reported, which successfully stained *Pythium* sp. in sections of the thallus of the macroalga *Porphyra* sp.

Lilley *et al.* (1997c) detected *A. invadans* in the tissue of EUS-infected fish with PABs so there was a strong possibility that one or more of the five MABs previously produced (Chapter 3) could be used in a similar way. The fact that several of the MABs recognised ECPs (Section 3.3.2) was of particular interest because if the ECPs are involved in pathogenicity, it is unlikely that the same antigens will occur in *Aphanomyces* spp. that are not fish pathogens.

Grocott's methenamine silver (GMS) stain for muco-polysaccharides (Section 2.2.3) is the most commonly used technique for detection of *A. invadans* in sections of EUS-infected fish. As it recognises all muco-polysaccharides, GMS is incapable of distinguishing between different species of fungi, even if they are taxonomically very different. Although invasive hyphae are characteristic of EUS, it cannot be assumed that invasive hyphae are invariably *A. invadans*. This problem is particularly acute when *A. invadans* first appears in a new geographical area, and researchers cannot be certain whether the observed clinical signs are due to *A. invadans* or an entirely new pathogen. The confusion surrounding the appearance of ulcerative mycosis (UM) in the USA (Section 1.1.1) illustrates the difficulties that may arise.

The present study began by optimising protocols for immunochemical labelling of *A. invadans* in pure cultures of germlings and tissue sections, using MABs raised to *A. invadans* (Chapter 3). The potential of the MABs for research and diagnosis was assessed by establishing the position of the antigens they recognised in the germlings, and by comparing the reaction of the MABs to *A. invadans* with their reaction to other oomycetes and other hyphal pathogens of aquatic animals. Results obtained with MABs were validated by comparing them with results obtained with GMS using tissue sections obtained from an experimental trial (Section 7.2.3).

4.2 Methods

4.2.1 Preparation of Materials

4.2.1.1 Preparation of Slides

Microscope slides (Surgipath Europe Ltd., Peterborough, UK) were treated with 3% v/v 3-aminopropyltriethoxysilane (APES) (Sigma) in acetone (Fisher) for 5min, then washed in acetone for 5min, followed by distilled water for 5min. After washing, the slides were air dried and stored at room temperature until they were used.

4.2.1.2 Preparations of Monoclonal Antibody Supernatants

Monoclonal antibody supernatants were prepared either directly from culture or concentrated by one of two methods. Concentrations of 20-30x were prepared by centrifuging the supernatants in concentrators with a molecular cut-off of 30kDa (Vivascience Inc., Westford, USA) at 3000g for 35min.

Concentrations of 100x were prepared by sodium sulphate precipitation (Bollag *et al.* 1996). MAb supernatants were heated to 30°C in a water bath, and 0.10M Na₂SO₄ (Sigma) was dissolved in them. The supernatants were centrifuged at 21000g at room temperature for 15min. Supernatants were discarded and the pellets were resuspended in 0.10M Na₂SO₄ in distilled water and centrifuged as before. After centrifuging, the supernatants were discarded again, the pellets were resuspended in more 0.10M Na₂SO₄ and the samples were centrifuged as before. After centrifuging, the pellets were dissolved in PBS to 1% of the original supernatant volume.

4.2.1.3 Preparation of Tissue Sections for Immunohistochemistry

Tissue sections of fish infected with EUS by bath challenge (Section 7.2.3) were prepared on APES-coated slides (Section 4.2.1.1), and dewaxed and rehydrated

(Section 2.2.2). The sections selected were from cases where hyphae were enclosed by distinct granulomata, and could be located by light microscopy without need for counter-staining.

4.2.1.4 Microscopy and Photography

Sections stained with fluorescein isothiocyanate (FITC) labelled secondary antibodies were viewed and photographed using an Olympus (Southend-on-Sea, UK) BX50 microscope and photographed with an Olympus SC35 camera with 800ISO 35mm film. Shutter speed was determined automatically and timed.

Viewing and photography of sections stained with peroxidase labelled secondary antibodies was carried out as for conventional stains (Section 2.2.3).

4.2.2 Assessment of Techniques for Immunocytochemistry

4.2.2.1 Preparation of Samples

For all methods, 1d PA8 germlings were adjusted to 10^5 germlings ml^{-1} in PBS and 40 μl of the suspension was placed in a hydrophobic enclosure on the slide drawn with a PAP pen (Sigma). The suspension was dried on to the slide at 40°C.

4.2.2.2 Antigen Retrieval

Antigen retrieval was attempted with one sample which was only tested with the most sensitive MAb, 3gJC9 (Section 4.3.1.2), and horse radish peroxidase (HRP) labelled secondary antibodies. The slides were placed in 0.05M citric acid at pH 6.0 and microwaved at 850W until the buffer boiled, then 150W for 8min in a Super Showerwave (Sanyo Fisher Company, Chatsworth, USA) microwave oven (Jung *et al.* 2001).

4.2.2.3 Immunocytochemistry with Peroxidase-Labelled Secondary Antibodies

A range of techniques were assessed for immunohistochemical labelling of formalin-fixed germlings.

The initial protocol was derived from Burr (1991), but several variations were assessed. First, endogenous peroxidase activity was removed by treating with 10% v/v H₂O₂ (Sigma) in methanol (Fisher) for 10 or 20min.

The slides were then washed with either TBS, Triton X-100 supplemented TBS (TBSX) (TBS containing 1% v/v Triton X-100 [Acros Organics, New Jersey, USA]), TTBS or PBS, by sluicing with the wash buffer, followed by covering each enclosure with the buffer for three 3 or 5min washes. All subsequent washes followed the same procedure.

Non-specific binding sites were blocked with 10% v/v goat serum in antibody buffer (Section 3.2.1) for 20min or 1h, and the slides washed again.

As much MAb supernatant as possible, usually 40-50µl, was added to each enclosure. The supernatant was incubated for 2h, 4h or overnight at the room temperature of 20-25°C or at 37°C. Positive controls were prepared with 0.01% v/v serum from the immunised mice in antibody buffer, and negative controls were prepared with antibody buffer alone. All three preparations of MAb supernatant were assessed.

After incubation with the MAb supernatant, the slides were washed and one of two possible secondary antibodies was applied. The first was 2% v/v HRP anti-mouse gamma globulin (Diagnostics Scotland) in antibody buffer for 1h. The second was 2% v/v biotin anti-mouse IgG (Diagnostics Scotland) in antibody buffer for 1.5h, which was followed by 2% streptavidin peroxidase (Diagnostics Scotland) in antibody buffer for 1.5h.

The slides were washed and developing solution was applied for 10min. Three developing solutions were assessed, True blue (Kirkregard & Perry Laboratories, Gaithersburg, USA), insoluble TMB from a peroxidase substrate kit (Vector Laboratories Inc., Burlingame, USA) or either 1.85M or 0.185M 3,3' diaminobenzidine tetrahydrochloride (DAB) (Sigma) and 0.0002% v/v H₂O₂ (Sigma) in PBS.

After development, slides were washed in running water for 10min, air dried at 40°C and cover slips were applied with Pertex (Cellpath).

4.2.2.4 Immunocytochemistry by Indirect Immunofluorescence Antibody Technique (IFAT)

The MAbs were evaluated with immunocytochemistry by IFAT, using secondary antibodies labelled with FITC instead of HRP. Slides and germlings were prepared in the same way as for other immunocytochemical methods. When the germlings had been dried on to the slide they were washed as previously described with PBS or TBSX. MAb supernatants and controls were applied as previously described and incubated for 2h, 4h or overnight at room temperature or at 37°C. MAbs concentrated by sodium sulphate precipitation were not assessed for IFAT.

After incubation, the slides were washed again and incubated with 2% v/v FITC labelled anti-mouse IgG (Diagnostics Scotland) in antibody buffer for 1h. The slides were washed again, and a drop of fluorescence mounting medium (DAKO Corporation, Carpinteria, USA) was added to each enclosure. Cover slips were applied and sealed with clear nail varnish.

The slides were immediately placed in the dark at 4°C and stored until they were viewed, less than 7d after preparation.

4.2.2.5 Multiple Reaction Cycling

Multiple reaction cycling (MRC) was used to enhance responses (Linsenmayer *et al.* 1988). Following the application of the secondary antibody, the slides were washed again and the MAb reapplied for 1h. After washing, the secondary antibody was reapplied and slides were washed and either developed or subjected to a third cycle.

MRC was assessed for immunocytochemistry with HRP labelled secondary antibodies and for IFAT.

4.2.3 Assessment of Techniques for IHC

4.2.3.1 IHC with Peroxidase-labelled Secondary Antibodies

IHC protocols were adapted from the most successful immunocytochemical technique (Section 4.3.1.1). The selected technique used an initial incubation in the MAb supernatant of 2h followed by 1h with HRP labelled secondary antibody. A second reaction cycle followed, which involved repeating the blocking step followed by 1h in MAb supernatant and 1h in secondary antibody solution. All washes were carried out with TBSX. The developing solution was 0.067% w/v DAB.

After the slides were developed and washed, they were counter-stained in 1% Mayer's haematoxylin (Section 2.2.4) for 4min, washed in running water for 5min, dehydrated and mounted (Section 2.2.4).

Two other protocols were assessed. One used a biotin labelled secondary antibody followed by streptavidin peroxidase as previously described.

The second method was the tyramine amplification technique (TAT) (von Wasielewski *et al.* 1997). Tyramide conjugate was prepared by mixing 500µl TAT solution A (18mM sulphosuccinimidyl-6-[biotinimide] hexanoate in dimethylformamide) with 1.445ml TAT solution B (10µl 7.2M triethylamine in

1.435ml 136mM dimethylformamide) and incubating in the dark for 2h. The resulting conjugate was diluted in 4.36ml ethanol and stored at 4°C (Hopman *et al.* 1998). TAT was carried out with a 1h incubation with biotin labelled secondary antibody, followed by 1h with streptavidin peroxidase. Tyramide conjugate was diluted at 0.001% v/v in TBS and incubated on the slides for 10min at 37°C. After a 30min incubation with streptavidin peroxidase, the slides were developed as previously described.

4.2.3.2 IHC by IFAT

A protocol for IHC by IFAT with MAb 3gJC9 was derived from the most successful technique for immunocytochemistry by IFAT (Section 4.3.1.1). Sections of infected fish from the passive immunisation trial (Section 7.3.3) were used for the test.

Slides were dewaxed and taken to water (Section 2.2.2). The sections were blocked with goat serum (Section 4.2.2.3) for 20min and washed by sluicing once followed by three 3min incubations in TBSX. All subsequent washes were done in the same way. The sections were enclosed in hydrophobic wax with a PAP pen and covered with neat MAb supernatant. Negative controls were covered with unused hybridoma supernatant. All slides were incubated overnight at 4°C in humidity chambers, which was found to give better results than incubations of either 1h or overnight at room temperature.

After washing, the sections were covered with 2% v/v FITC labelled anti-mouse IgG for 1h at room temperature. The slides were washed again and a second reaction cycle of blocking and application of the MAb and secondary antibody with intermediate washes was carried out. After a final wash, the sections were covered by 0.5% w/v methyl green (Hopkin & Williams Ltd, Chadwell Heath, UK) in PBS for 5min at room temperature to reduce background fluorescence (Lannan *et al.* 1991). They were then washed in running water for 5min.

The sections were covered with fluorescence mounting medium, and cover slips were applied and sealed with clear nail varnish. The slides were stored in the dark at 4°C to dry, and examined no more than 5d after preparation.

The optimised technique was repeated using the other four MAbs, using sections prepared from the same tissue block in order to compare them to MAb 3gJC9.

4.2.4 Evaluation of IHC

4.2.4.1 Cross-Reactivity to Other Oomycetes

The specificity of the MAbs was tested by comparing their reaction to PA8 germlings by IFAT immunocytochemistry with their reaction to various other oomycete germlings.

Slides were prepared with duplicate positive controls of PA8 and duplicate treatment and negative controls of the test isolate. Unconcentrated MAb supernatants were added to positive controls and treatments, and unused hybridoma culture medium was added to negative controls.

Incubation times were those found to be optimal in the development of IFAT, namely 4h for MAb supernatants and 2h for secondary antibodies.

When slides were viewed, the reaction in the treatment enclosures was ranked according to its intensity when compared to the positive and negative controls (Beakes *et al.* 1997).

4.2.4.2 Comparison with Response to Similar Diseases

Sections of fish infected with other hyphal pathogens of fish (Table 4.1) were stained with the optimised protocol for IHC by IFAT (Section 4.3.1.2, Appendix II) with MAb 3gJC9 to establish whether there was any cross-reaction with them.

Sections of tissue from plague-infected crayfish were included in the study as the results obtained with pure cultures indicated that MAb 3gJC9 cross-reacted with *A. astaci* (Table 4.3). The results of staining with one, two and three reaction cycles were assessed using section 1247/96/II from a plague-infected crayfish.

Table 4.1. Tissue sections of aquatic organisms with hyphal diseases other than EUS used to assess cross-reactivity of MAb 3gJC9.

Section reference	Host species	Pathogen	Disease	Supplied by
5950020U	Striped snakehead <i>Channa striata</i>	<i>Saprolegnia</i> sp. isolate TF29	Injection challenge	Dr. J.H. Lilley
500-007SKIN	Channel catfish <i>Ictalurus punctatus</i>	<i>Saprolegnia</i> sp.	Saprolegniasis	Dr. L. Khoo & Dr. A. Grooters
R970036B	Atlantic salmon <i>Salmo salar</i>	<i>Exophiala</i> sp.	Exophialasis	Dr. R. Collins
599-1070-1	Channel catfish <i>Ictalurus punctatus</i>	<i>Branchiomyces</i> sp.	Branchiomycosis	Dr. L. Khoo & Dr. A. Grooters
82-1240	Atlantic menhaden <i>Brevoortia tyrannus</i>	Unknown	Ulcerative mycosis	Dr. E.J. Noga
1247/96/II	Noble crayfish <i>Astacus astacus</i>	<i>Aphanomyces</i> <i>astaci</i>	Crayfish plague	Dr. B. Oidtmann
629/96/c	Noble crayfish <i>Astacus astacus</i>	<i>Aphanomyces</i> <i>astaci</i>	Crayfish plague	Dr. B. Oidtmann

4.2.4.3 Comparison of IHC with GMS

All samples from the passive immunisation and immersion challenge trial (Section 7.3.3) were stained with MAb 3gJC9 using the optimised IHC by IFAT protocol (Section 4.3.1.2, Appendix II), and the presence or absence of *A. invadans* hyphae was noted. The results were compared to those obtained by GMS (Section 2.2.3) to establish which method was more sensitive.

4.3 Results

4.3.1 Optimisation of Protocols

4.3.1.1 Immunocytochemistry

The most sensitive method among those assessed for immunocytochemistry was indirect IFAT, using unconcentrated MAb supernatant incubated for 4h, followed by the secondary antibody incubated for 2h, at room temperature. The slides were washed by sluicing with PBS followed by three 5min incubations with PBS.

Immunocytochemistry with peroxidase-labelled secondary antibodies was only successful using MAb 3gJC9 with any protocol. The most successful protocol used a 3h incubation with unconcentrated MAb supernatant followed by 1h with HRP-labelled secondary antibodies. A second reaction cycle followed, incubating for 1h with MAb supernatant and 1h with the secondary antibody before development with 1.85M DAB. Washing was carried out by sluicing with TBSX, then three 3min incubations in the same solution.

Neither concentrating the antibody solutions nor antigen retrieval improved the results of staining in any way. Use of a biotin labelled secondary antibody and streptavidin peroxidase, or TMB or true blue as peroxidase substrates, gave such high background reactions so that it was difficult or impossible to distinguish samples treated with MAb supernatants from negative controls.

4.3.1.2 Immunohistochemistry

The most successful technique for IHC was indirect IFAT, although some modification was needed from the immunocytochemistry protocol and clear results were only obtained with MAb 3gJC9.

The technique described for immunocytochemistry with peroxidase labelled secondary antibodies proved mildly successful for IHC using MAb 3gJC9, though it was necessary to repeat the blocking step in the second reaction cycle to prevent excessive background staining and the DAB concentration was lowered to 0.185M to reduce background. Use of biotin labelled secondary antibodies and streptavidin peroxidase increased background, and the signal was only improved when TAT was used (Fig. 4.1). No protocol using peroxidase-labelled secondary antibodies was able to

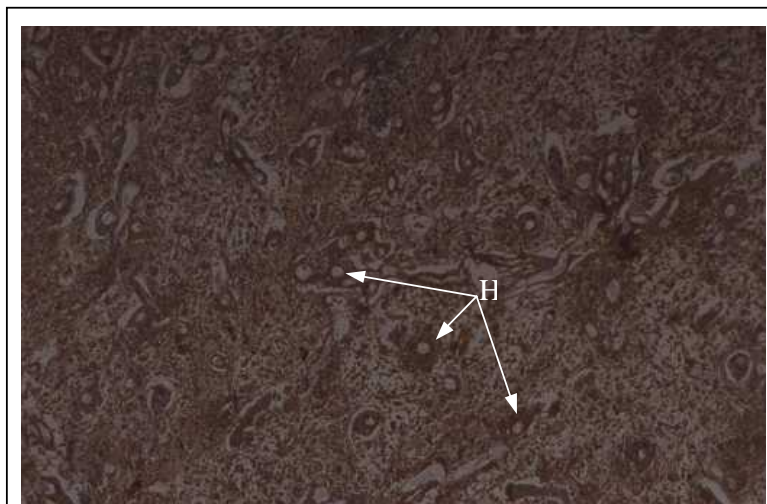


Fig. 4.1. Section of EUS-infected snakehead muscle tissue stained with MAb 3gJC9 and enhanced with TAT, followed by haematoxylin counter staining, illustrating hyphae (H). 100x magnification.

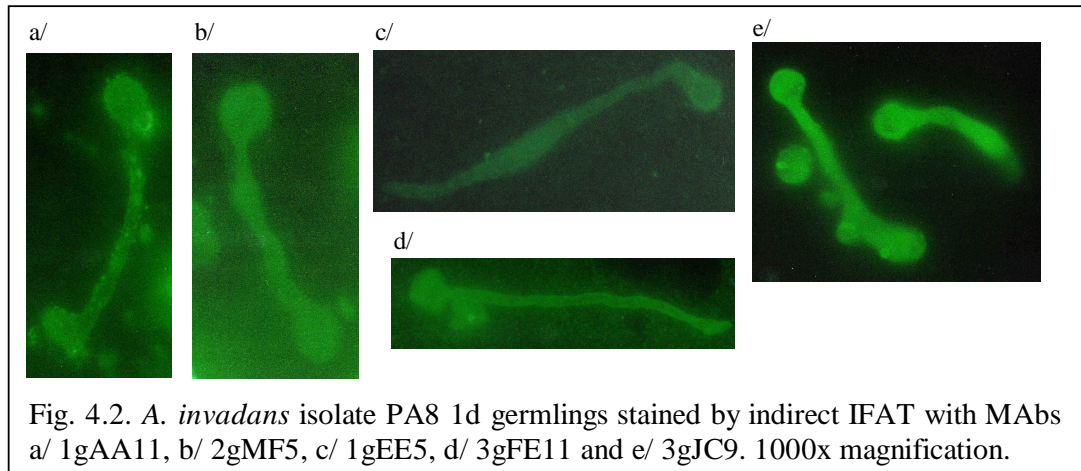
match the clarity or contrast with background that was obtained by indirect IFAT.

A step-by-step protocol for the optimised IHC technique is given in Appendix II.

4.3.2 Observations Derived from Immunochemical Stains

4.3.2.1 *Aphanomyces invadans* Germlings

All antibodies induced stronger fluorescence at the heads of the germlings than the germ tubes. MAb 1gAA11 recognised distinct parts of the germ tube, but all the others induced uniform fluorescence across it. The fluorescence was very faint in the case of MAb 2gMF5. MAb 3gJC9 induced strong fluorescence from the germ tubes of shorter germlings that were evidently at an early stage of development (Fig. 4.2).



4.3.2.2 EUS-Infected Fish

No reaction was apparent when MABs 2gMF5 and 1gAA11 were used. MABs 1gEE5 and 3gFE11 both highlighted the hyphae when the optimised indirect IFAT protocol (Section 4.3.1.2) was used, but contrast was poor and detailed observations were not possible.

Sections stained with MAB 3gJC9 exhibited strong fluorescence around the hyphae (Fig. 4.3a,b). Most of the fluorescence was concentrated in a rim around the hyphae, although the surrounding tissue also fluoresced, particularly where it was heavily necrotic. Well developed granulomata appeared to concentrate the fluorescence much closer to the hyphae. The hyphal protoplasm only fluoresced at what appeared to be the very ends of the hyphae (Fig.4.3f), which were rarely observed. There was very little fluorescence observed in the region of undamaged muscle tissue in uninfected fish, or away from the locus of infection in infected fish.

At the periphery of the infected site, pinpoints of fluorescence were observed around the hyphae (Fig. 4.3b), often extending into parts of the sections that still contained intact muscle fibres. In some cases, developed hyphae were absent or only found in the dermis, and only the sources of pinpoint fluorescence appeared in the

musculature (Fig 4.3a). At higher magnifications, the pinpoints appeared filamentous, but no clear photographs were obtained at magnifications of above x40.

In sections treated with hybridoma culture medium as a negative control, there was a generally lower level of background fluorescence. In some cases, fluorescence was observed in sharply defined rings, which probably indicated the cell walls of the hyphae. This non-specific fluorescence was more noticeable in sections that had been treated with three reaction cycles, and less evident in sections that had only been treated with two. It was easily distinguishable from sections stained with MAb 3gJC9.

4.3.2.3 Comparison of IFAT with GMS

Tissue sections collected from an immersion challenge (Section 7.3.3) were stained with GMS and the optimised IHC technique using MAb 3gJC9.

In most cases, the results of both protocols agreed. However, there were several cases where positive results were recorded from IHC on sections where *A. invadans* did not appear to be present after GMS (Table 4.2). In all such cases, developed hyphae similar to those recognised by GMS were either absent or confined to the dermis, where they did not appear to be invasive, but the aforementioned pinpoint fluorescence sources indicated a diagnosis of EUS. No equivalent of the pinpoint sources of fluorescence observed using IFAT (Section 4.3.2.2) was observed (Fig. 4.3).

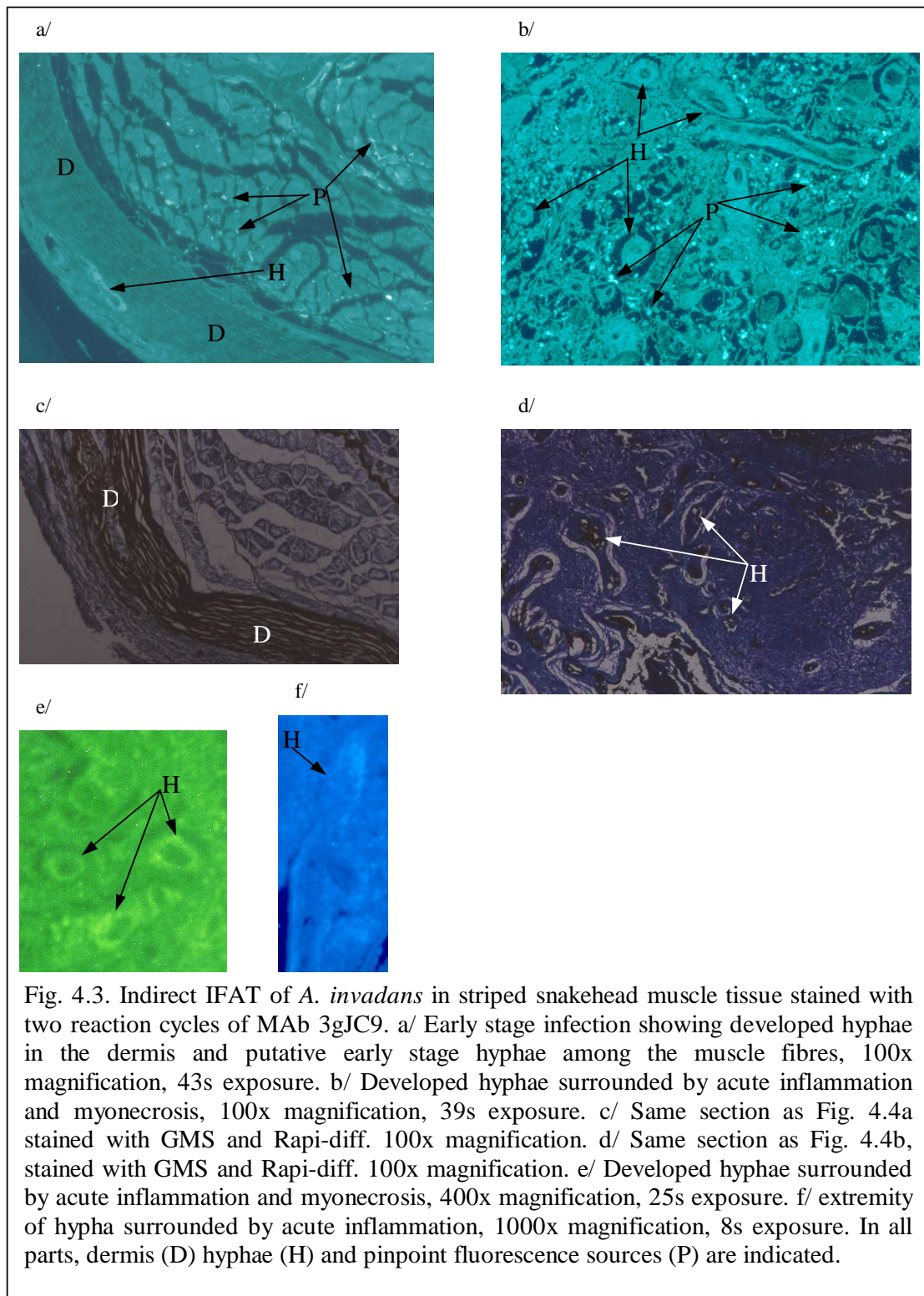


Table 4.2. Incidence of EUS in fish challenged with *A. invadans* zoospores after passive immunisation with three different sera assessed by IFAT and GMS.

	Serum 1		Serum 2		Serum 3	
	IFAT	GMS	IFAT	GMS	IFAT	GMS
1	x	x	x	x	x	x
2	x	x	*	x	x	x
3	x	x	x	x	✓	✓
4	x	x	x	x	✓	✓
5	*	x	x	x	✓	✓
6	✓	x	✓	✓	*	x
7	x	x	x	x	✓	✓
8	✓	x	x	x	✓	✓
9	x	x	✓	✓	x	x
10	x	x	✓	✓	*	✓
11	✓	✓	✓	✓	x	x
12	✓	✓	✓	✓	✓	✓
13	✓	✓	✓	✓	✓	✓
14	✓	✓	✓	✓	✓	✓
15	✓	✓	✓	✓	✓	✓
16	x	x	x	x	x	x

✓ indicates positive for EUS

x indicates negative for EUS

* indicates presence of putative early stage infection

4.3.3 Cross-Reactivity

4.3.3.1 Cross-Reactivity to Other Oomycete Germlings

All the MAbs reacted with all the *A. invadans* isolates tested, though they all cross-reacted with at least one other *Aphanomyces* sp. (Table 4.3). MAb 3gJC9, which gave the clearest results when used for IHC (Section 4.3.1.2), only cross reacted with *A. astaci*.

4.3.3.2 Comparison with Response to Similar Diseases

The optimised IHC protocol was carried out on several hyphal diseases other than EUS to assess its specificity as a diagnostic tool.

The mycelium did not fluoresce any more strongly than the background in the case of sections of fish infected with *Saprolegnia* spp, *Exophiala* sp. or *Branchiomyces* sp. The section of the fish with UM contained invasive hyphae that fluoresced strongly, in a manner indistinguishable to sections of fish with EUS (Fig 4.4).

Sections of plague-infected crayfish did not induce as strong a reaction as sections of EUS-infected fish. No fluorescence was observed in the crayfish sections after one IHC cycle. After two cycles, the mycelium fluoresced slightly more strongly than the background tissue, and after three cycles, strong fluorescence was detectable (Fig 4.5).

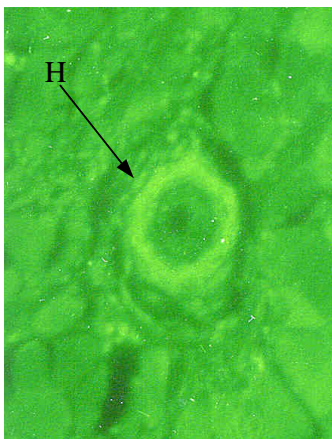


Fig. 4.4. Tissue of UM infected Atlantic menhaden stained with two cycles of MAb 3gJC9. 400x magnification, 4s exposure.

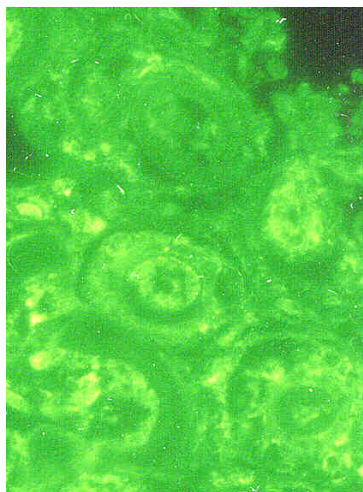


Fig. 4.5. Section of noble crayfish infected with crayfish plague, stained with three cycles of MAb 3gJC9. 400x magnification, 22s exposure.

Table 4.3. Cross reactivity of MAbs against *Aphanomyces invadans* isolate PA8 1d germlings with 1d germlings of other oomycetes.

Isolate	Species	Description	MAbs				
			1gAA11	1gEE5	2gMF5	3gFE11	3gJC9
B99C	<i>Aphanomyces invadans</i>	EUS pathogen	+	+	+	+	+
T99G2	<i>Aphanomyces invadans</i>	EUS pathogen	+	+	+	+	-
UM3	<i>Aphanomyces invadans</i>	UM pathogen	+	+	+	+	-
FDL458	<i>Aphanomyces astaci</i>	Crayfish plague pathogen	-	--	--	--	-
ASEAN1	<i>Aphanomyces laevis</i>	Plant pathogen	--	--	-	-	--
SA11	<i>Aphanomyces</i> sp.	non-invasive wound pathogen	++	-	-	--	--
84-1249	<i>Aphanomyces</i> sp.	non-invasive UM isolate	--	--	--	--	--
99ExtAph	<i>Aphanomyces</i> sp.	non-invasive wound pathogen	--	--	--	-	--
ACHLYA99	<i>Achlya</i> sp.	non-invasive wound pathogen	--	--	--	--	--
TF23	<i>Saprolegnia</i> sp.	non-invasive wound pathogen	--	--	-	--	--
P32	<i>Saprolegnia ferax</i>	Saprophyte	--	-	--	--	--
795	<i>Saprolegnia australis</i>	Saprophyte	-	-	--	--	--
TP41	<i>Saprolegnia parasitica</i>	Saprolegniasis pathogen	-	-	--	--	--
E3	<i>Saprolegnia diclina</i>	Saprophyte	+	+	-	--	--
3501d	<i>Leptolegnia caudata</i>	Insect pathogen	--	--	-	--	--
--			Fluorescence intensity indistinguishable from negative control				
-			Fluorescence intensity between negative and PA8				
+			Fluorescence intensity indistinguishable from PA8				
++			Fluorescence intensity greater than PA8				

4.4 Discussion

The MAbs raised to *A. invadans* germlings were assessed in terms of their potential for IHC diagnostic methods. All five MAbs recognised all the *A. invadans* isolates when the optimised immunocytochemistry technique was applied to germlings. MAbs 1gAA11, 1gEE5, 2gMF5 and 3gFE11 cross-reacted with other *Aphanomyces* spp., and all but 3gFE11 cross-reacted with oomycetes from other genera. MAb 3gJC9 only cross-reacted with *A. astaci* and was the only MAb that gave reliable and repeatable results, so most of the IHC was carried out with MAb 3gJC9.

All of the MAbs recognised antigens on the heads of the germlings, and all except MAb 1gAA11 recognised antigens that were evenly distributed across the surface of the germ tube. The fact that the antigens were on the surface was expected as the mice were immunised with whole germlings (Section 3.2.2.2). Western blots had already established that MAbs 2gMF5, 3gFE11 and 3gJC9 reacted with the same antigen (Chapter 3), and these results were corroborated by similar staining of germlings. The small number of bands recognised by MAb 2gMF5 by Western blot analysis was reflected by the weak fluorescence induced in its reaction to germlings, suggesting that its affinity for its antigen is very low.

All of the MAbs reacted with *Aphanomyces* isolate UM3, isolated from a UM-infected fish, as strongly as they did with other *A. invadans* isolates. Such uniformity was not found in the reactions to any other oomycetes, which supports the conclusion of Blazer *et al.* (1999) that UM is caused by *A. invadans*.

Similarly, the tissue section of an Atlantic menhaden with UM appeared identical to sections with EUS when stained with MAb 3gJC9. This finding supports the early conclusions from research on UM that the most important cause was an oomycete pathogen, now considered to be *A. invadans* (Noga & Dykstra 1986, Noga *et al.* 1988,

Blazer *et al.* 1999). None of the MAbs cross-reacted with *Aphanomyces* isolate 84-1249, which was isolated from an early UM outbreak but did not reproduce the pathology of UM (Dykstra *et al.* 1986, Noga 1993b). This conclusion is supported by the fact that isolate 84-1249 was not recognised by *A. invadans*-specific PCR probes (Panyawachira *et al.* 1999). The combination of reactivity to isolate UM3 and the hyphae in a UM infected fish, but not to isolate 84-1249, suggests that 84-1249 was an opportunistic wound pathogen rather than a causative agent of UM.

The association of *A. invadans* with UM still begs several questions. Environmental stress factors are usually associated with EUS (Section 1.1.4), so they are probably also associated with UM. Kane *et al.* (2000) suggested that algal blooms may be such a factor, but the interactions between algal blooms and *A. invadans* infection in UM have not been studied. Another enigma is that menhaden with UM lesions have been sampled from water with salinities of up to 20‰ (Kane *et al.* 2000), which is considerably higher than the lethal salinity for *A. invadans* (Fraser *et al.* 1992).

The difficulty in obtaining species-specific antibodies to *Aphanomyces* spp. (Petersen *et al.* 1996, Lilley *et al.* 1997c) was reflected in the fact that all the MAbs cross-reacted with at least one other isolate from the genus. *Aphanomyces* isolate SA11 cross-reacted with three of the MAbs, one of them more strongly than *A. invadans*, although it is unclear why it was so much more reactive than the other *Aphanomyces* saprophytes. *Saprolegnia diclina* isolate E3 also caused considerable unexplained cross-reactivity, although there is some ambiguity about the classification of E3 as RFLP revealed considerable differences between E3 and other *S. diclina* isolates (Molina *et al.* 1995).

Most MAbs used by fish pathologists (Adams *et al.* 1995) and PABs raised to *A. invadans* (Lilley *et al.* 1997c) label their targets when fairly standard protocols are

applied (Harlow & Lane 1988, Anderson 1990, Liddell & Cryer 1991). Although such techniques were used for immunocytochemistry of *A. invadans*, it was necessary to attempt several recently developed techniques intended for use against rare antigens (Mayer & Bendayan 2001) before a reliable IHC protocol could be developed. Two such techniques, namely TAT (von Wasielewski *et al.* 1997) and MRC of indirect IFAT (Linsenmayer *et al.* 1988), were successful. The latter was selected for further development as it labelled the hyphae far more conspicuously.

All of the antibodies produced in the present study were of the IgM isotype. The pentameric form of IgM is unstable (Draber *et al.* 1995), and it is possible that disruption of the pentamer may adversely effect its binding abilities. The lack of stability may be responsible for the poor response observed if only one cycle was used. While the first cycle relies on the MAb binding to the antigen, the second cycle involves the MAb being bound by the hitherto unbound site on the secondary antibodies bound to the first cycle MAbs (Linsenmayer *et al.* 1988). The binding of the secondary antibody to IgM molecules in the second cycle is probably unaffected by whether or not the molecule is part of a complete pentamer or not, which may explain the considerable improvement in the response induced by a second cycle.

The antigen recognised by MAb 3gJC9 was a protein that occurred mainly in the ECPs (Chapter 3). Its cross-reactivity with *A. astaci*, but not with non-invasive pathogens or saprophytes, strongly suggests that the antigen may be involved in the pathogenicity of both species, which are closely related taxonomically (Hart 1998).

It is likely that ECPs involved in the virulence of a crustacean pathogen would perform similarly if expressed by a fish pathogen, explaining why *A. astaci* and *A. invadans* apparently express the same antigen in their ECPs. It cannot be assumed that this finding indicates a more general homogeneity in the ECPs of the two species, as

Lilley (1997) found that *A. astaci* does not infect fish and *A. invadans* does not infect crayfish.

The fact that the antigen is an ECP accounts for the observation that fluorescence is not restricted to the hyphae themselves but also appeared in host tissue. Where the host tissue is inflamed and necrotic, the antigen appears to be diffused throughout much of the tissue, often some distance away from the hyphae. It is likely that much of the necrosis is actually caused by the ECPs, which pathogens such as *A. astaci* use to break up tissue and allow penetration of hyphae (Söderhäll & Unestam 1975, Söderhäll *et al.* 1978).

The fluorescence was much less diffuse where the hyphae were enclosed by granulomata. The granulomata probably prevented the spread of the ECPs, limiting the damage that they caused. Containment of virulence related ECPs by granulomata may explain why the rapid development of granulomata is often associated with resistance to EUS (Chinabut *et al.* 1995, Wada *et al.* 1996, Chapter 6).

It is not clear what is indicated by the pinpoints of fluorescence that MAb 3gJC9 recognised in apparently undamaged muscle tissue, where there was little evidence of the presence of ECPs. At a higher magnification, some of the points were filamentous and some resembled germinating cysts, but it is more likely that the similarity was coincidental as it is unlikely that the cyst would penetrate the muscle tissue before germinating. It is far more likely that they represent a very early stage of mycelial development that has not been previously observed. Their fluorescence was denser than on the mature hyphae, which may be due to the early stage having a less developed cell wall. Such an early stage may be constructed more similarly to the germings that the MAbs were raised against than the mature hyphal wall, and MAb 3gJC9 appeared to bind to it in a similar way to its uniform binding to the membrane of the germling.

These stages were not clear when stained with GMS, so the many studies that have relied on GMS would not have reported this stage. Lilley *et al.* (1997c) used PABs raised to ECPs for IHC and made no mention of unusually narrow hyphae, but the pinpoint fluorescence was not recorded in every sample and may not have been present for the samples used in that study.

Like the diffuse ECPs, the pinpoint sources were not found where granulomata had developed, which further supports the suggestion that they were an early infective stage, restricted to the active periphery of the spreading mycelium.

It must be stressed that the observations on pathology made in the present study were drawn from a trial that was not intended primarily as a histopathological study (Chapter 7). Ideally, fish should have been sampled for histopathology at regular intervals after challenge. Similarly, while it appears likely that the pinpoint fluorescence sources are early stages of the mycelium, this cannot be firmly concluded and electron microscopy would be needed to describe them thoroughly.

The only MAb likely to be of any use in diagnostics is MAb 3gJC9, as all the others gave only weak reactions with poor contrast, or no reactions at all, when applied to *A. invadans* in tissue sections. Every section stained positive by GMS also stained positive by IFAT with MAb 3gJC9. Several sections that were recorded as not containing *A. invadans* by GMS were also positive according to IHC. In all such cases, mature hyphae were confined to the dermis if present at all, and only the apparent early stage of the mycelium had penetrated into the muscle tissue. When the sections were stained with GMS, the section did not conform to the case definition of EUS (Roberts *et al.* 1994) as the early stage mycelium was not clear, there were no granulomata and hyphae in the dermis were considered to be non-invasive.

All of the sampled fish were moribund at the time of sampling, and it is unlikely that as mild an infection as was observed in these cases could have reduced the fish to such a state. It is possible that such an infection in the sampled section was actually only the periphery of a more serious infection in parts of the fish that were not sampled. The fish had been acid stressed and scraped (Section 7.2.3.3) before challenge so it is also possible some may have contacted a more rapidly spreading opportunistic infection. Infection with *A. invadans* may have facilitated infection by an opportunist or exacerbated its seriousness, as opportunistic bacteria have been described as a major cause of death in EUS outbreaks (Boonyaratpalin 1989).

Regardless of the fact that *A. invadans* may not have been the main cause of pathology, IHC was evidently a more sensitive test for its presence than GMS. Even if the pinpoint fluorescence sources cannot be considered conclusive evidence of the presence of *A. invadans* in themselves, the fact that MAb 3gJC9 did not cross-react with known opportunistic saprophytes enables its presence to be confirmed by the presence of hyphae in the dermis. This is not possible with sections stained with GMS, where the presence of opportunistic saprophytes obscures any *A. invadans* hyphae that may be present.

The Uvitex stain for cellulose is commonly used to detect oomycetes (Wachsmuth 1988), and has recently been advocated for the diagnosis of EUS (Yorisada *et al.* 1999). Uvitex was not considered in the present study, but it is more rapid and requires less costly reagents than either GMS or IHC. A further advantage of the Uvitex stain over IHC is that it may be followed by H&E to enable more detailed observation of pathology. Uvitex is currently the stain of choice at AAHRI, the OIE diagnostic centre for EUS (Section 1.1.5), although Uvitex does not have the specificity of IHC.

A final possible application for MAb 3gJC9 is suggested by the fact that it cross-reacted with *A. astaci* germings. The three reaction cycles necessarily for reliable labelling are not unprecedented, as some immunohistochemical protocols recommend up to four cycles to enhance fluorescence (Linsenmayer *et al.* 1988, Mayer & Bendayan 2001). It is also possible that the lower reactivity in tissue sections than the reactivity with pure cultures suggested was related to the fact that it was not possible to obtain sections that had not been decalcified, which may have disrupted antigens. However, only two crayfish sections were available and the protocol was not optimised for crayfish plague due to time constraints, so the potential of MAb 3gJC9 for the study of crayfish plague was not thoroughly explored.

4.5 Summary

- All the MAbs raised to *A. invadans* germings recognised all the *A. invadans* isolates tested.
- MAbs 1gAA11, 1gEE5, 2gMF5 and 3gFE11 cross-reacted with several other oomycetes and showed poor antigen binding, so their potential for immunodiagnostics was not considered in depth.
- MAb 3gJC9 only cross reacted with *A. astaci*, and showed the strongest binding characteristics, so was selected for IHC.
- The clearest IHC results were obtained by two cycle indirect IFAT with MAb 3gJC9.
- A structure considered to be an earlier stage of the mycelium than has previously been reported was revealed by IHC.
- IHC was more sensitive than GMS, as MAb 3gJC9 detected antigens in sections that were not stained by GMS.

- A section of an Atlantic menhaden from the USA with UM stained identically to striped snakeheads with EUS, suggesting that UM and EUS are synonymous.

5 Interaction of Macrophages and Serum of EUS-Susceptible and Resistant Fish with *Aphanomyces invadans**

5.1 Introduction

In order to find possible methods of enhancing the immune response to EUS, it is necessary to establish how *A. invadans* interacts with the host. The differences in susceptibility within and between species (Section 1.1.4) suggest that host responses are far from straightforward.

Most previous studies on the immune response to *A. invadans* have focused on the striped snakehead, which is commonly reported as highly susceptible (e.g. Tonguthai 1985, Bhaumik *et al.* 1991). However, susceptibility is reduced in subsequent outbreaks (Chinabut 1998), indicating an improvement in the mechanisms of defence. Chinabut *et al.* (1995) showed that supposedly susceptible snakeheads were resistant to new infections and recovered from existing infections when the temperature was raised above 26°C, indicating that effective immune responses may be present, but are suppressed at low temperature.

On the other hand, some species such as common carp (Wada *et al.* 1996) and Nile tilapia (Khan *et al.* 1998) are completely immune to EUS at all temperatures. Major differences between species have also been reported in responses of crustaceans

* Studies described in this chapter have been published as:

Miles, D.J.C., Seetubtim, P., Lilley, J.H., Kanchanakhan, S., Thompson, K.D., Chinabut, S. & Adams, A. (1999). The *in vitro* effect of macrophages and serum on the germination of *Aphanomyces invadans*. Fourth Symposium on Diseases in Asian Aquaculture, Cebu City, Philippines, 22-26 November, 1999.

Miles, D.J.C., Kanchanakhan, S., Lilley, J.H., Thompson, K.D., Chinabut, S. & Adams, A. (2001). Effect of macrophages and serum of fish susceptible or resistant to epizootic ulcerative syndrome (EUS) on the EUS pathogen, *Aphanomyces invadans*. *Fish & Shellfish Immunology*. In press.

to *A. astaci* (Section 1.4.4), and while resistant signal crayfish showed a faster melanotic response than susceptible noble crayfish (Unestam & Weiss 1970), no complete explanation for the difference has ever been offered.

The interactions of human granulocytes with fungal pathogens such as *Candida albicans* (Diamond *et al.* 1978), *Trichophyton rubrum* (Dahl & Carpenter 1986) and *Aspergillus fumigatus* (Diamond & Clark 1982, Levitz *et al.* 1986) have been widely studied *in vitro*. In all cases, granulocytes surrounded the hyphae and inhibited growth.

Granuloma formation is a common response of fish to invasive pathogens (Secombes & Fletcher 1992), and is so frequently associated with EUS that it was incorporated into the case definition (Roberts *et al.* 1994). Rapid granuloma formation has been associated with the immunity of common carp to EUS (Wada *et al.* 1996) and the improved immunity of striped snakeheads at high temperatures (Chinabut *et al.* 1995).

The mechanism by which the granulocytes inhibit the invasive hyphae is unclear, though the superoxide anion (O_2^-), which is produced by the respiratory burst reaction of granulocytes, is employed in the immune responses of many animals and plants (Sutherland 1991). Superoxide is involved in a range of responses to hyphal pathogens as diverse as that of human macrophages to *A. fumigatus* (Diamond & Clark 1982) and that of potato tubers to the oomycete, *Phytophthora infestans* (Doke 1983).

Superoxide production is not the only mechanism by which granulocytes have been observed to interact with fungal pathogens. For example Diamond *et al.* (1978) found that human neutrophils attacked *C. albicans* by releasing lysozyme. Nevertheless, the strength of the respiratory burst of head-kidney macrophages *in vitro* is a widely used indicator of immunocompetance in fish, especially when provoked by stimulants such as 4- α -phorbol 12-myristate 13-acetate (PMA) (Secombes 1990).

Humoral components of the innate immune system have also been associated with resistance to fungi. The alternative complement pathway (ACP) is involved in resistance to many fungal pathogens of mammals (Lehmann 1985). Teleost complement proteins differ very little from those of mammals (Nonaka *et al.* 1984), and Ji *et al.* (1997) suggested that complement proteins are conserved throughout the chordates. Although the role of fish ACP in immunity to fungi and oomycetes has not been previously investigated, such a similarity of form suggests a similarity of function.

Complement has been implicated in differences in immunity to specific pathogens between closely related species. The thorny skate *Amblyraja* (= *Raja*) *radiata* and the cuckoo ray *Leucoraja* (= *Raja*) *naevus* are closely related and share the same habitat, but the latter is commonly infested by the cestode *Acanthobothrium quadripartitum*, which is not found to be associated with the former. The results of studies *in vitro* indicated a mechanism for the attachment of thorny ray complement to *A. quadripatitum* that was not available in cuckoo ray complement, explaining the considerable difference in susceptibility between the two species (McVicar & Fletcher 1970).

Specific antibody responses to hyphal infection of mammals are commonplace (Casadevall 1995, Romani & Howard 1995), though they have only been conclusively proven to be protective in a few cases such as that of horses to the oomycete, *Pythium insidiosum* (Mendoza *et al.* 1992b) and the dermatophyte, *Trichophyton equinum* (Pier & Zancanella 1993). In fish, antibodies to *A. invadans* have been found in striped snakehead (Thompson *et al.* 1997) and rainbow trout (Thompson *et al.* 1999), though whether they are protective is unclear.

The present study compared the mechanisms by which EUS-susceptible and non-susceptible fish respond to *A. invadans* by comparing the interactions of the

macrophages and serum of such species with *A. invadans* *in vitro*. It also investigated the possibility of attenuation of such mechanisms at the low temperatures at which outbreaks typically occur.

5.2 Methods

5.2.1 Experimental fish

All experimental fish were from central Thailand (Table 5.1), where EUS had regularly been reported for 17 years preceding the study (Tonguthai 1985).

Table 5.1. Species, source, weight and EUS-susceptibility of fish.

Species	Source	Mean weight (g)	sd of weight	EUS-Susceptibility [*]
Striped snakehead <i>Channa striata</i> (Bloch) (Perciformes: Channidae)	Pond farms, Suphanburi Province	87.2	22.3	Yes
Silver barb <i>Barbodes</i> (= <i>Puntius</i>) <i>gonionotus</i> (Bleeker) (Cypriniformes: Cyprinidae)	National Aquaculture and Genetic Research Institute, Patum Thani Province	198.8	68.1	Yes
Giant gourami <i>Osphronemus gouramy</i> Lacepède (Perciformes: Osphronemidae)	Pond farms, Uthai Thani Province	609.6	219.1	Yes
Swamp eel <i>Monopterus</i> (= <i>Fluta</i>) <i>albus</i> (Zouiev) (Synbranchidae: Synbranchiformes)	Wild caught fish from Saphanmai market, Bangkok	152.4	28.0	Yes
Nile tilapia <i>Oreochromis niloticus</i> (Linnaeus) (Perciformes: Cichlidae)	Singburi Province Fisheries Station.	95.9	25.8	No

sd = standard deviation

^{*} Lilley *et al* (1998)

Fish from all five species were acclimatised to 20°C (± 2) for at least three weeks. Other snakeheads, silver barbs and tilapia were acclimatised to the ambient temperature of approximately 32°C (± 5) at which EUS is rarely reported. All fish were fed twice daily on Charoen Pokphand (CP) (Bangkok, Thailand) brand no. 9910 pellets, supplemented with wild caught, chopped snails in the case of swamp eels. Water quality was maintained by partial water changes twice weekly. All fish were kept in 150l glass tanks.

It was not possible to use all individuals for every analysis due to difficulties with bacterial contamination of some samples, but all analyses involved a minimum of five and a maximum of twelve individuals per group.

5.2.2 Experimental pathogen

All experiments were carried out using the cysts of isolate NJM9701 (Section 2.1.3). It had been isolated two years previous to the experiment and pathogenicity had recently been confirmed by injection into striped snakeheads in a separate experiment (Dr. J.H. Lilley & Miss. V. Panyawachira, unpublished data).

5.2.3 Reagents

All solutions were prepared in distilled water unless otherwise specified. All reagents were obtained from Sigma (Poole, UK) unless otherwise specified.

Benzocaine solution was 0.73M benzocaine in absolute ethanol.

For macrophage isolation, isolation medium was Leibowitz L-15 medium containing 1000U ml⁻¹ penicillin, 1mg ml⁻¹ streptomycin, 100U ml⁻¹ heparin and 20ml l⁻¹ inactivated FBS (Section 3.2.1). Washing medium was L-15 containing 1000U ml⁻¹ penicillin, 1mg ml⁻¹ streptomycin and 1ml l⁻¹ inactivated FBS. Percoll gradients were made by layering 34% Percoll (distilled water containing 34% v/v Percoll, 10% 10x v/v

minimum essential medium) on top of 51% Percoll (distilled water containing 51% v/v Percoll, 10% v/v 10x minimum essential medium). Maintenance medium was L-15 medium prepared at double concentration from powder, containing 50ml l⁻¹ inactivated FBS for all treatments.

For the respiratory burst assay, nitro blue tetrazolium (NBT) solution (1.22mM NBT in Hanks' balanced salts solution), and PMA-NBT solution (1.62µm PMA in NBT solution) were prepared.

Lysis buffer was 100mM citric acid containing 1% v/v Tween 20 and 0.1% w/v crystal violet, sterile filtered at 0.45µm.

To prepare antigen for ELISA, Wood's (1988) extraction buffer was prepared using 85mM trizma base, 1mM magnesium chloride, 1mM EDTA, 10mM potassium chloride, 1.12mM ascorbic acid, 10.9mM glycerol, pH 7.5 and 5µM phenylmethanesulphonylfluoride (PMSF) to inhibit endogenous protease activity.

All solutions are described in Appendix I.

5.2.4 Collection of serum and macrophages

Fish were killed by overdose of benzocaine solution. As much blood as possible was immediately taken from the caudal vein. It was allowed to clot for 2h at 25°C (± 1.0), centrifuged at 10,000g for 15min and serum was pipetted off. Fungicidal assays were prepared immediately and serum for assessment of relative antibody concentration was stored at -70°C until the assay was performed.

The macrophage isolation protocol was adapted from Secombes (1990), using sterile materials and techniques throughout. It was not performed with swamp eels as preliminary experiments failed to isolate macrophages from the kidney.

Head kidneys were dissected out and placed in isolation medium. The head kidneys were macerated through a 100µm mesh into more isolation medium, layered on top of a Percoll density gradient and centrifuged at 400g. Macrophages were collected from the gradient layer and washed by centrifuging twice in washing medium at 900g. Viable macrophages were counted by mixing an aliquot of macrophage suspension with an equal volume of 0.1% v/v trypan blue in PBS and counting on an improved Neubauer haemocytometer. Macrophages were adjusted to 10^6 cells ml⁻¹ for fungicidal assays and 10^7 cells ml⁻¹ for respiratory burst assays with washing medium. Aliquots of 100µl were seeded into 96-well microplates and incubated for 2h at 23°C ± 1, which preliminary studies had shown to be optimal for all species used in the study. Non-adherent leukocytes were removed with three washes of L-15 pre-warmed to the incubation temperature and the culture wells were loaded with 100µl maintenance medium.

Duplicate wells were seeded for assessment of number of viable cells after the assays were completed in both fungicidal and respiratory burst assays.

5.2.5 Fungicidal assays

Triplicate wells per treatment were prepared with 100µl maintenance medium.

Cysts were prepared on the day the fish were sampled (Section 2.1.3), counted in an improved Neubauer haemocytometer and adjusted to a concentration of 4.0×10^4 ml⁻¹. Aliquots of 50µl were added to triplicate wells containing macrophages, 20µl serum, 20µl serum heated at 55°C for 30min to inactivate complement or control wells containing medium only. The volume of every well was made up to 200µl with APW, giving a final cyst concentration of 10^4 ml⁻¹ (Thompson *et al.* 1999).

After 24h, germination in each well was assessed by counting visible germlings within one microscope field of view at 40x magnification, and extrapolating the count to the total area of the well for comparison between treatments and control. Germling growth was assessed by measuring ten randomly chosen germlings from each well using a graticule.

A mucus fungicidal assay was attempted, using mucus collected by shaking the freshly killed fish in a plastic bag sterilised by ultra-violet (UV) light for 30min. The mucus was scraped off the inside of the bag with an autoclaved spatula (Wood *et al* 1988). Triplicate wells of a 96-well microplate were prepared with 100µl germedia, 20µl mucus and 30µl APW, and UV-sterilised for 30min. 50µl of cyst suspension was added, and the assay was incubated and assessed in the same way as the serum fungicidal assays. However, it was found that in spite of the sterilisation procedure, a high concentration of motile bacteria were almost always present in the wells at the time of assessment, and no meaningful results were obtained by this method.

5.2.6 Respiratory burst assay

Three groups of triplicate wells were seeded with macrophages. One group was inoculated with 10^4 fungal cysts ml^{-1} and all were incubated for 20h. Other triplicate wells were seeded only with fungal cysts to confirm that the germlings themselves had no effect on optical density.

After incubation, intracellular superoxide production was evaluated using the NBT assay (Secombes 1990). PMA-NBT solution was added to one group of uninoculated wells, and NBT solution to all others. After 30min, the NBT and PMA-NBT solutions were removed and the reaction was stopped with absolute methanol. Wells were washed three times with 70% methanol in distilled water. The resulting formazan was dissolved in 120µl 2M potassium hydroxide and 140µl DMSO per well,

and the optical density was measured in a multiscan spectrophotometer at a wavelength of 610nm.

To calibrate the results of the assays, serial doubling dilutions were made of a high but unknown concentration of superoxide, generated from the highest possible concentration of giant gourami macrophages. A standard curve of absorbance against superoxide concentration in arbitrary units was prepared, and shown to be extremely precise ($r^2 = 0.99$). All subsequent measurements were compared to the standard and results were converted into a superoxide concentration in arbitrary units 10^5 macrophages⁻¹.

5.2.7 Assessment of Macrophage Adherence

Macrophage adherence was assessed by counting the number of macrophages attached to the wells set aside for that purpose, at the time the fungicidal assay was assessed or the respiratory burst assay was carried out.

Counts were carried out by removing the medium, washing three times with L-15 to remove non-adherent macrophages from the wells and incubating in lysis solution pre-cooled to 4°C, for 10min. The contents of the wells were mixed and macrophage nuclei were counted with an improved Neubauer haemocytometer.

5.2.8 Assessment of relative antibody concentration (RAC)

Relative anti-*A. invadans* antibody concentrations (RACs) were quantified by ELISA.

Antigen was prepared by grinding 3d germlings in liquid nitrogen, thawing and collecting in extraction buffer. Protein concentration was assayed with a Bio-Rad (Hercules, USA) protein assay, adjusted to $1\mu\text{g ml}^{-1}$ in PBS and stored at -70°C.

MAB supernatants were collected (Section 3.2.2) from cultures of hybridoma cell lines 7D2 raised against striped snakehead immunoglobulin, 5G11C2 against giant

gourami immunoglobulin and M40 against Nile tilapia immunoglobulin. No anti-silver barb or anti-swamp eel immunoglobulin MAb was available.

The ELISA was adapted from Chen *et al.* (1996). Hydrophilic Immulon 4 HBX ELISA plates (Dynex Technologies Inc., Chantilly, USA) were coated, loaded with antigen and blocked (Section 3.2.3.2). Serial doubling dilutions of test serum in antibody buffer were prepared from 1/8 to 1/4096 in duplicate per individual fish. Duplicate negative controls were prepared by adding antibody buffer only. Duplicate positive control wells were prepared with a 1/100 dilution of serum of the species tested previously shown to be positive. After 2h, the plates were washed, incubated with secondary antibodies and developed (Section 3.2.3.2).

Antibody was considered to be present if mean test serum absorbance exceeded three times that of the negative control at any dilution. For the purposes of comparison, a mean negative control was calculated and the readings for each individual were corrected for differences in background absorbance by subtracting the difference of the mean negative control for that individual from the overall mean negative control. Adjusted absorbance was plotted against dilution for each individual and RAC was calculated for each by reading the absorbance at the midpoint of the dilutions (Harlow & Lane 1988).

5.2.9 Statistical analysis

Initial analysis on the growth and germination inhibition assays was carried out by comparing the results from treatment and control wells by 2-way analysis of variance (ANOVA) with interaction, followed by Tukey multiple comparisons. Treatments were designated as fixed factors and individual fish as random factors. It was not possible to transform the data from the germination inhibition assay of low temperature snakeheads

to conform to the assumptions of ANOVA, so a Kruskal-Wallis test with Dunnett C multiple comparisons was used instead.

Further analyses were carried out to establish whether there were differences between those treatments in which growth or germination was significantly different to controls. Comparisons were made between fish of the same species kept at different temperatures, or between fish of different species kept at the same temperature. Each treatment count was converted into an index of inhibition by dividing it by the mean control value so that indices greater than one indicated reduction of germination and indices below one indicated increased germination. Indices of inhibition by serum and heated serum were compared by nested ANOVA with the results for individuals nested within species or temperature groups, followed by Bonferroni multiple comparisons. Indices of inhibition by macrophages were similarly nested, and the macrophage counts were used as covariates for nested analysis of covariance (ANCOVA).

Where there were differences in inhibitory activity between heated and unheated serum, the magnitudes of those differences were compared by a similar nested ANOVA with Bonferroni multiple comparisons.

The mean macrophage count in the fungicidal assays at the time of assessment was compared between species at the same temperature by 1-way ANOVA and Tukey multiple comparisons. Counts were compared between temperature groups of the same species by independent samples t-tests.

Macrophage counts in the fungicidal assays were plotted against superoxide production by both treatments and control macrophages in the respiratory burst assays.

The superoxide production of PMA stimulated macrophages was compared to that of control macrophages by 2-way ANOVA with interaction, with treatment and

individual as the factors. The effect of incubation with *A. invadans* was similarly compared to the same controls.

Three measures of superoxide production in the inhibition assay were calculated for each individual by dividing the superoxide production per 10^5 macrophages from each of the three treatments in the respiratory burst assay by 10^5 , and multiplying by the number of macrophages in the inhibition assay. Indices of inhibition were plotted against all the adjusted measurements of superoxide production to establish whether there was any covariance. Where present, the covariance was evaluated by regression analysis.

It was not possible to compare RAC between species as the MAbs used to assess them were not standardised. Comparison between individuals of the same species at different temperatures was carried out by independent samples t-tests.

Covariance between RAC and inhibition of germination and growth was assessed by plotting mean indices of inhibition against relative antibody concentration for each fish. Critical values of $p < 0.05$ were accepted as significant in all tests. Test statistics are presented where results refer to only one test. Where results refer to more than one test, the critical value given refers to the highest present in any case.

5.3 Results

5.3.1 Factors inhibiting germination of *A. invadans*

Macrophages of all groups other than snakeheads reduced germination ($p < 0.05$). Sera of all groups other than swamp eels and low temperature tilapia reduced germination ($p < 0.05$). High temperature snakeheads were the only group in which heated sera reduced germination ($q = 5.09$, $df = 18.49$, $p < 0.05$), and that of tilapia at either temperature increased it ($p < 0.05$) (Table 5.2).

In all cases where sera inhibited germination, heated sera had no effect or stimulated growth except in the case of high temperature snakeheads where heated sera was inhibitory although less so than unheated sera ($q = 8.70$, $df = 18.49$, $p < 0.001$). In the two cases where sera had no effect, swamp eel heated sera also had no effect ($F = 2.044$, $df = 2$, $p > 0.05$) but low temperature tilapia heated sera stimulated growth ($q = 7.92$, $df = 18.02$, $p < 0.001$).

Table 5.2. Factors that significantly ($p < 0.05$) affected the germination of *A. invadans* cysts in comparison to control cysts.

Species	Temperature	Serum	Heated serum	Macrophages
Striped snakehead	Low	↓	x	x
<i>Channa striata</i>	High	↓	↓	x
Silver barb	Low	↓	x	↓
<i>Barbodes gonionotus</i>	High	↓	x	↓
Giant gourami	Low	↓	x	↓
<i>Osphronemus gouramy</i>				
Swamp eel	Low	x	x	-
<i>Monopterus albus</i>				
Nile tilapia	Low	x	↑	↓
<i>Oreochromis niloticus</i>	High	↓	↑	↓

- = not measured
 ↓ = less germination than controls
 ↑ = more germination than controls
 x = no difference to controls

5.3.2 Factors inhibiting growth of *A. invadans*

Silver barb and gourami macrophages, serum or heated serum reduced germeling growth ($p < 0.01$). Swamp eel sera also inhibited growth ($q = 116.97$, $df = 12.07$, $p < 0.001$). The heated sera of swamp eels, high temperature snakeheads and low temperature tilapia increased germeling growth ($p < 0.05$) (Table 5.3).

Germelings were shorter when treated with low temperature silver barb heated serum than with unheated serum ($q = 4.14$, $df = 20$, $p < 0.005$), but there was no

difference in the effect of any other case where both treatments reduced growth ($p > 0.05$).

Table 5.3. Factors that significantly ($p < 0.05$) affected the growth of *A. invadans* germings in comparison to control germings.

	Temperature	Serum	Heated serum	Macrophages
Striped snakeheads <i>Channa striata</i>	Low	x	x	x
	High	x	↑	x
Silver barbs <i>Puntius gonionotus</i>	Low	↓	↓	↓
	High	↓	↓	↓
Giant gourami <i>Osphronemus gouramy</i>	Low	↓	↓	↓
Swamp eels <i>Monopterus albus</i>	Low	↓	↑	-
Nile tilapia <i>Oreochromis niloticus</i>	Low	x	↑	x
	High	x	x	x

- = not measured

↓ = less germination than controls

↑ = more germination than controls

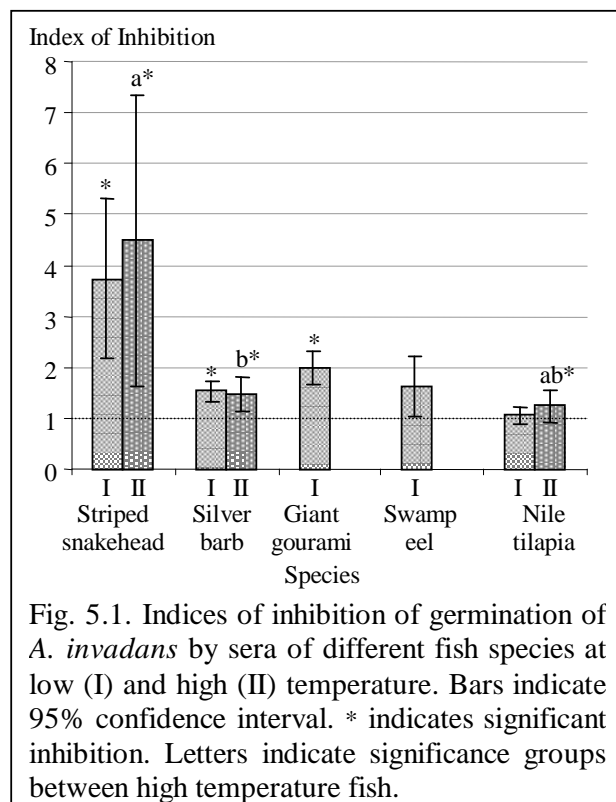
x = no difference to controls

5.3.3 Comparisons of the effect of macrophages from different species and temperature groups on germination and growth

No differences in macrophage activity on fungal germination or growth were found between groups other than those described above ($p > 0.05$).

5.3.4 Comparisons of the effects of sera and heated sera from different species and temperature groups on germination and growth

At low temperature, there were no significant differences between the activity of sera of different species on germination other than those described above ($F = 2.17$, $df = 2$, $p > 0.05$). At high temperature, snakehead sera were more inhibitory than those of silver barbs ($t = 2.66$, $df = 17$, $p < 0.05$), though not those of tilapia ($t = 1.90$, $df = 17$, $p >$

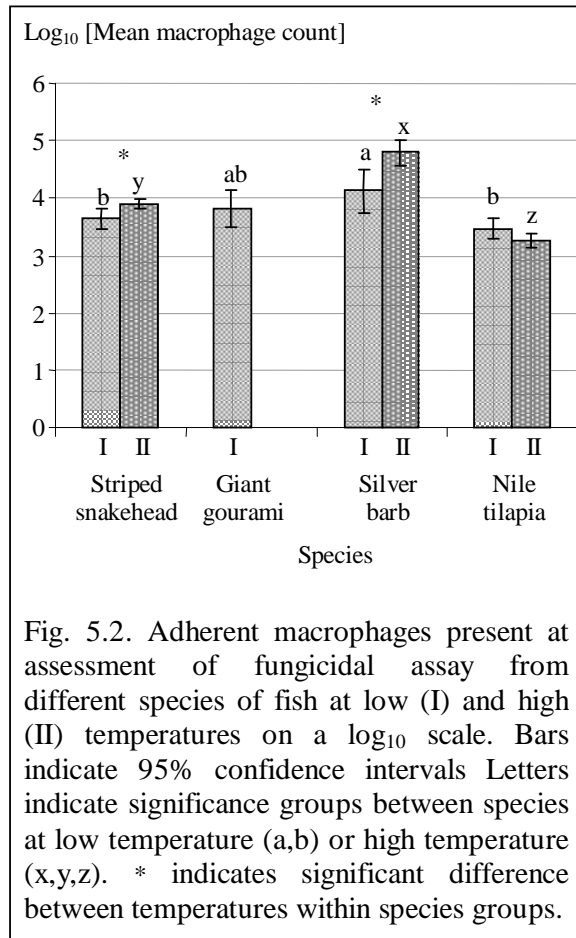


0.05), and tilapia sera inhibition were not different to silver barb sera inhibition ($t = 0.60$, $df = 17$, $p > 0.05$) (Fig. 5.1).

No significant differences ($p > 0.05$) in the effects of heated sera on germination, sera or heated sera on germling growth, or the effect of heating serum, were found between groups other than those described above (Sections 5.3.1, 5.3.2, Table 5.2, Table 5.3).

5.3.5 Comparisons of proportions of macrophages recovered

Among fish at low temperature, there were significantly more silver barb macrophages than snakehead ($q = 2.90$, $df = 27$, $p < 0.05$) or tilapia ($q = 3.03$, $df = 27$, $p < 0.05$) macrophages counted in the fungicidal assay, but no other differences ($p > 0.05$).



tilapia at different temperatures ($t = 1.92$, $df = 9$, $p > 0.05$) (Fig. 5.2).

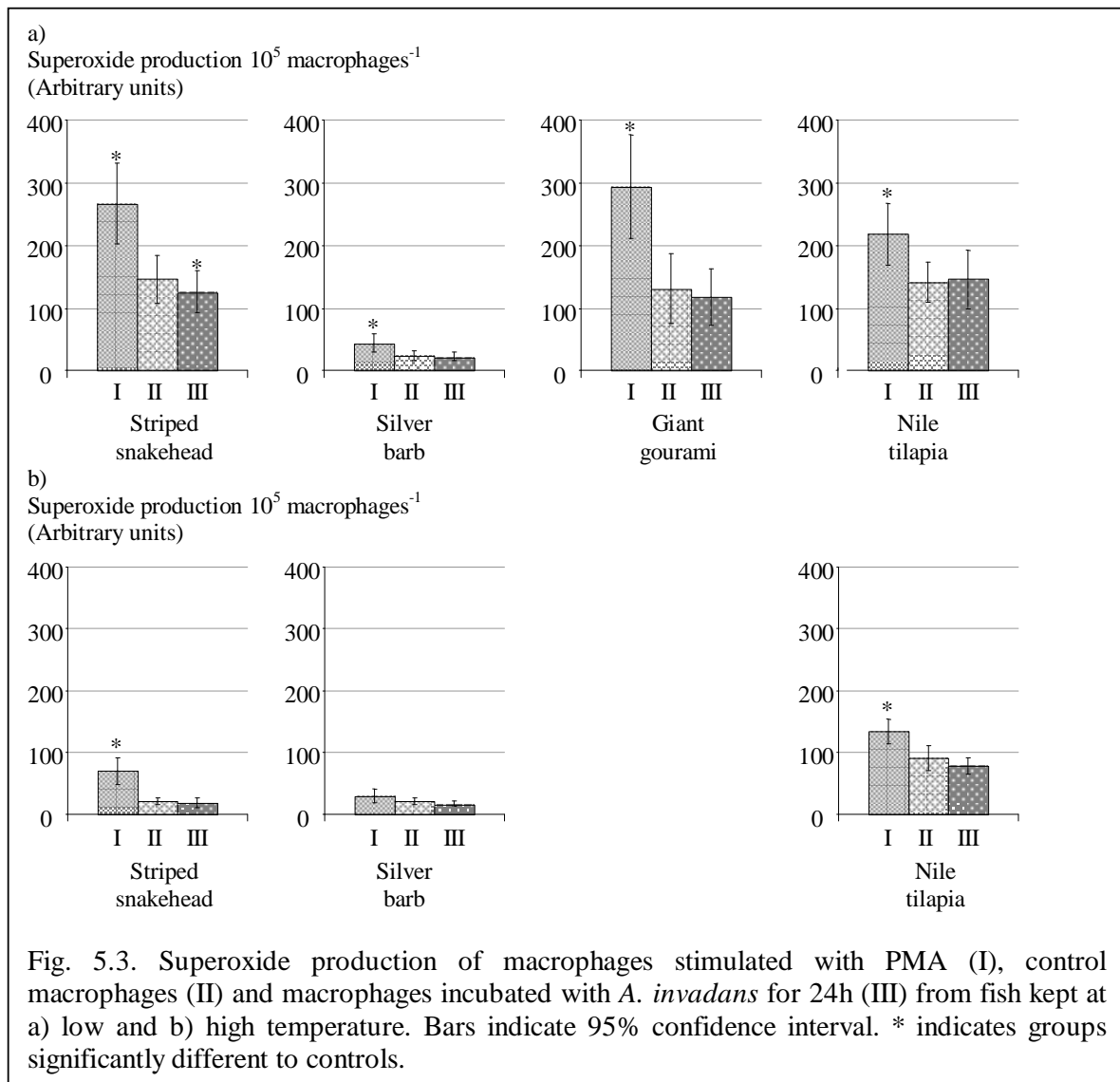
No covariance between superoxide production and macrophage adherence was found.

5.3.6 Effect of PMA and *A. invadans* on intracellular respiratory burst

The PMA-stimulated macrophages produced significantly ($p < 0.0005$) more superoxide than control macrophages in all cases except that of high temperature silver barbs ($F = 5.16$, $df = 1$, $p > 0.05$). Control macrophages produced more superoxide than inoculated macrophages in all cases other than low temperature tilapia, although the difference was only significant in the case of snakeheads at either temperature ($p < 0.05$) (Fig. 5.3).

At high temperature, there were more adherent silver barb macrophages than of either of the other two species, ($p < 0.0005$) and more adherent snakehead macrophages than tilapia macrophages ($q = 6.34$, $df = 21$, $p < 0.0005$).

There were more adherent macrophages from high temperature snakeheads ($t = 3.35$, $df = 8$, $p < 0.05$) and silver barbs ($t = 3.02$, $df = 10$, $p < 0.05$) than low temperature fish of the same species. No differences were found between



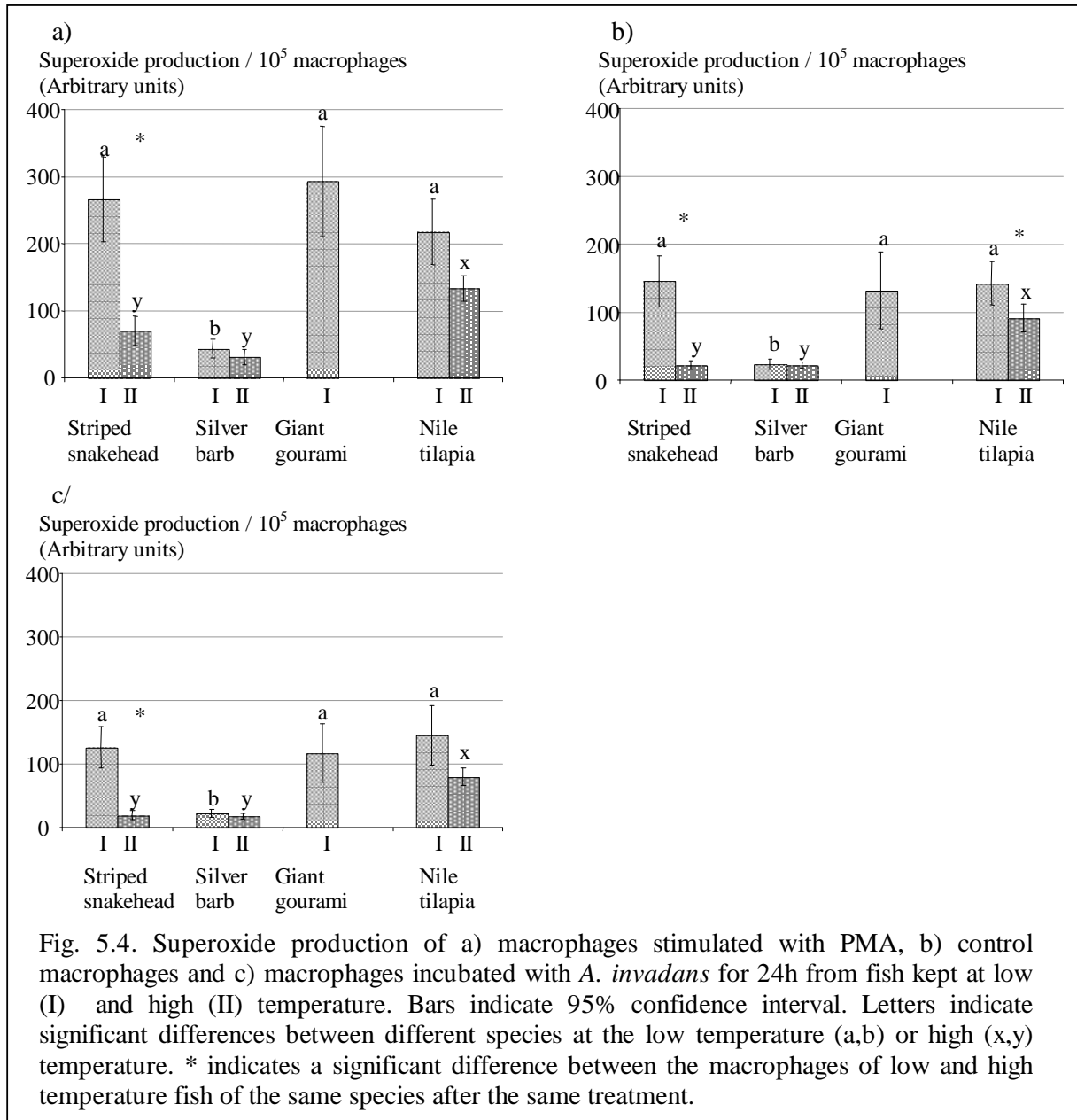
5.3.7 Comparisons of intracellular respiratory burst between groups

Among low temperature fish, silver barb macrophages had the lowest respiratory burst in all three treatments ($p < 0.05$). There were no other significant differences.

At high temperature, there were no differences between barb and snakehead macrophages ($p > 0.05$), but tilapia macrophages produced more superoxide in all treatments ($p < 0.05$).

Respiratory burst was higher at low temperature in all groups and treatments, though the differences were not significant in any treatment of silver barb macrophages

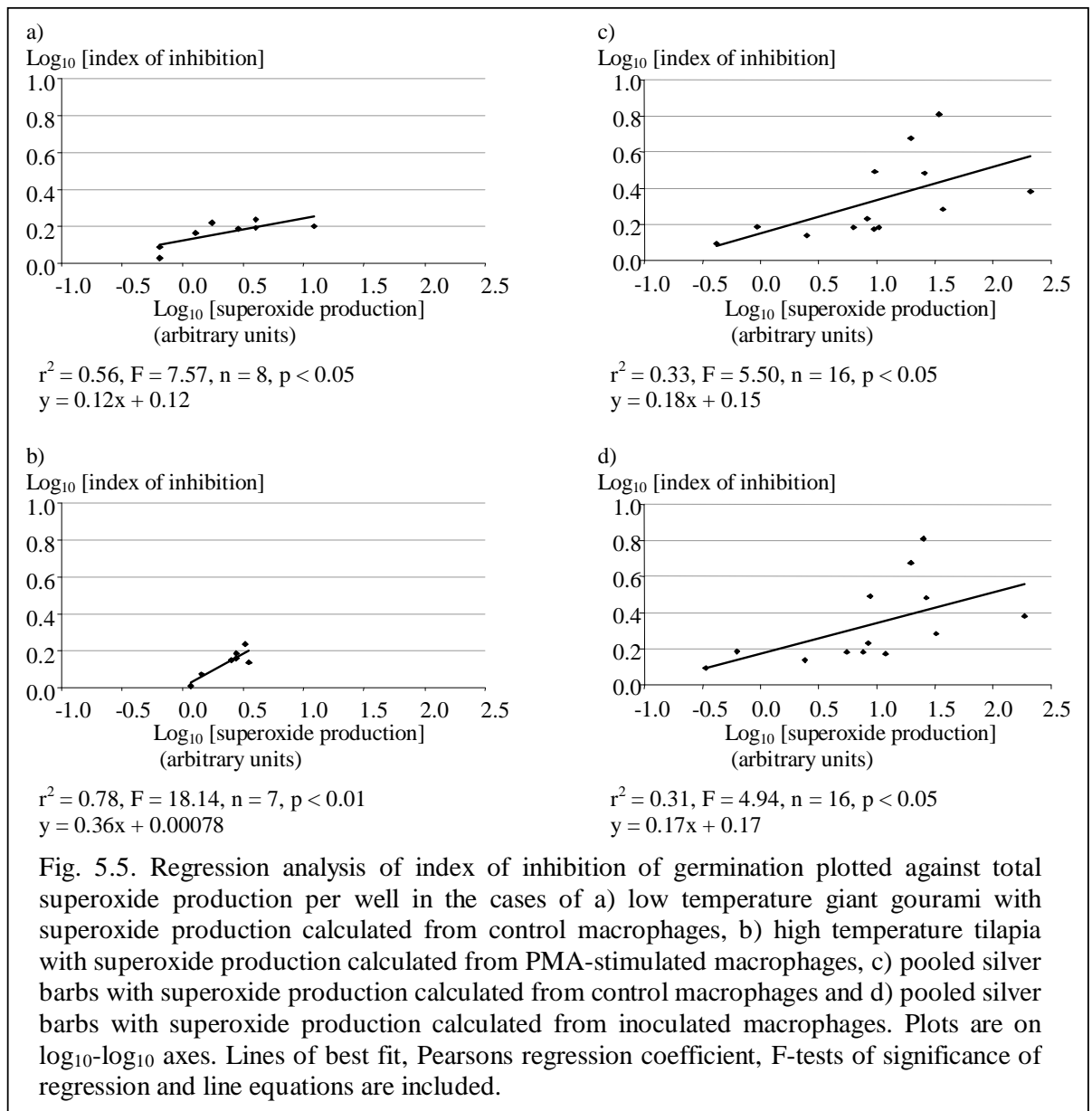
($p > 0.05$), and in the case of tilapia, were only significant in control macrophages ($F = 0.74$, $df = 1$, $p < 0.0005$). All three treatments of snakehead macrophages showed significant difference between temperatures ($p < 0.01$) (Fig. 5.4).



5.3.8 Relationship between respiratory burst and inhibition of germination by macrophages

An assessment of respiratory burst based on control macrophages showed a positive relationship in the case of high temperature tilapia with superoxide production derived from PMA-stimulated macrophages ($r^2 = 0.78$, $F = 18.14$, $df = 1$, $p < 0.01$), and low temperature gourami with superoxide data derived from control macrophages ($r^2 = 0.56$, $F = 7.57$, $df = 1$, $p < 0.05$) (Fig. 5.5).

Superoxide production data from both temperature groups of silver barbs, which



did not vary between temperature groups (Fig. 5.4), were pooled. Weak positive regressions were obtained from data derived from control ($r^2 = 0.33$, $F = 5.50$, $df = 1$, $p < 0.05$) and inoculated macrophages ($r^2 = 0.31$, $F = 4.94$, $df = 1$, $p < 0.05$) (Fig. 5.5).

5.3.9 Relationship between respiratory burst and inhibition of growth by macrophages

Low temperature snakeheads gave positive quadratic regressions with superoxide data derived from PMA-stimulated ($r^2 = 0.95$, $F = 51.61$, $df = 1$, $p < 0.0005$), inoculated ($r^2 = 0.86$, $F = 7.94$, $df = 1$, $p < 0.01$) and control ($r^2 = 0.76$, $F = 14.80$, $df = 1$, $p < 0.05$) macrophages. The only other group to give significant regressions was that of low temperature gourami, where control macrophages gave a negative linear relationship ($r^2 = 0.80$, $F = 23.69$, $df = 1$, $p < 0.005$) and inoculated macrophages gave a negative quadratic relationship ($r^2 = 0.74$, $F = 7.01$, $df = 1$, $p < 0.05$) (Fig. 5.6).

5.3.10 RAC

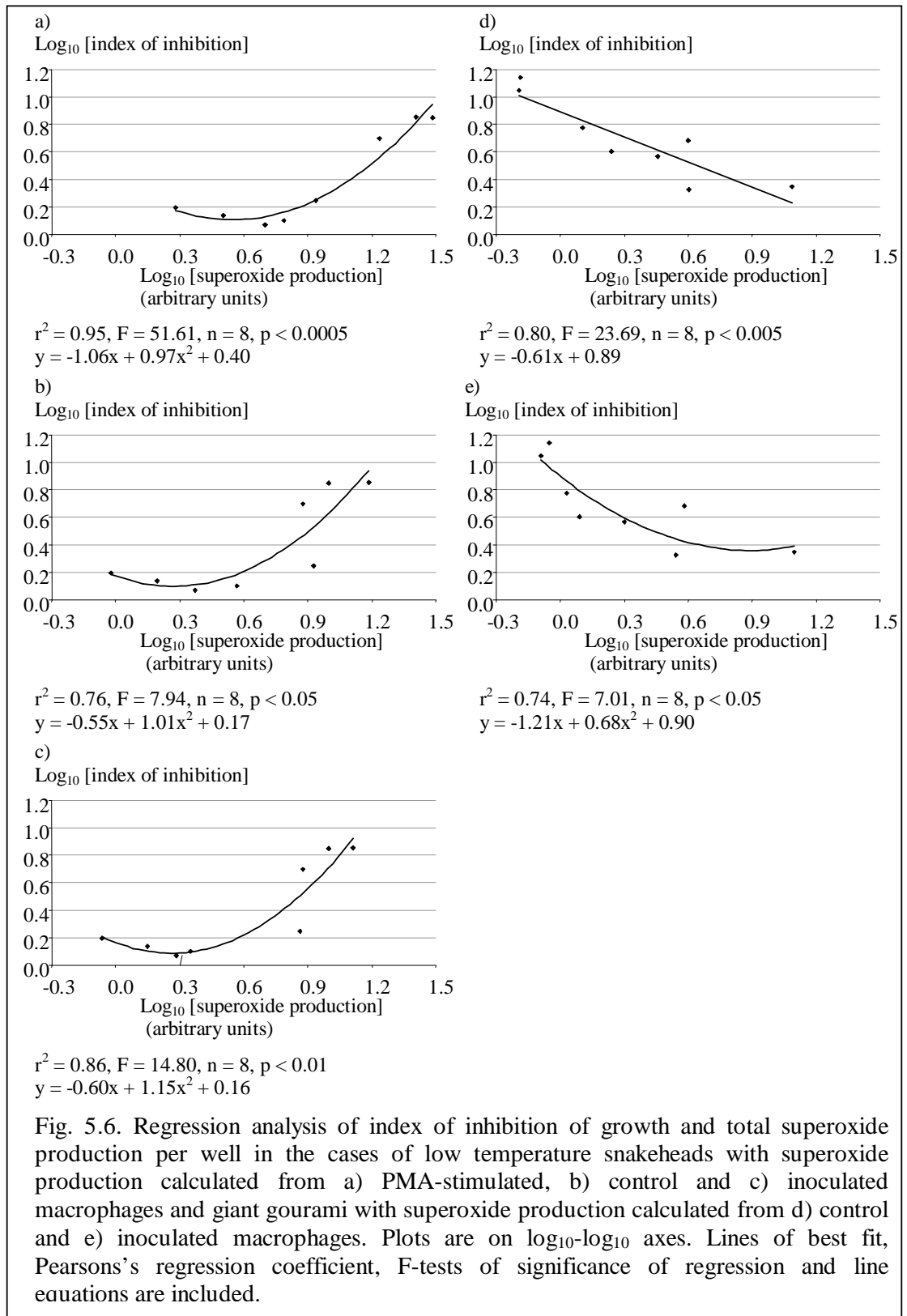
All snakeheads, gourami and high temperature tilapia had anti-*A. invadans* antibodies. Seven of the eight low temperature tilapia examined had anti-*A. invadans* antibodies.

High temperature snakeheads and tilapia had higher RAC than low temperature snakeheads and tilapia respectively ($p < 0.05$) (Fig. 5.7). No covariance between RAC and inhibitory activity was found in any case.

5.4 Discussion

The anti-*A. invadans* activity of the macrophages and serum of EUS-susceptible fish was compared to that of non-susceptible fish at EUS-permissive and non-permissive temperatures.

The lack of inhibition by the macrophages of highly susceptible snakeheads indicates that the inflammatory response that forms in the early stages of infection is



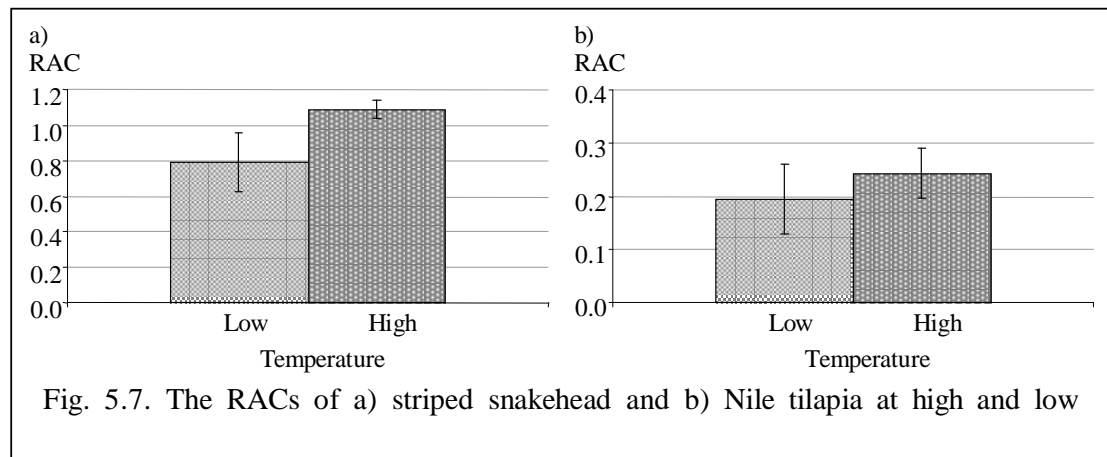


Fig. 5.7. The RACs of a) striped snakehead and b) Nile tilapia at high and low

probably ineffective at preventing the spread of infection in that species. By contrast, the inhibitory properties of serum equalled or exceeded those of the other species in all cases.

Silver barbs and gourami were the only groups in which serum, heated serum and macrophages all inhibited growth. Kurata *et al.* (2000b) found that the serum of the common carp, a cyprinid like the silver barb, had similar anti-fungal properties. It is possible that a similar factor is present in silver barbs, though its chemical nature remains unknown.

The activity of swamp eel sera was unusual, as it was unable to inhibit germination but could inhibit growth, in contrast to other susceptible species examined that could all inhibit germination. Further, swamp eel sera stimulated growth when heated, while sera of other species that inhibited growth continued to inhibit growth after heating. These inconsistencies suggest that swamp eel sera interacts with *A. invadans* using completely different mechanisms to the other species, which is unremarkable given the substantial anatomical and taxonomic differences between swamp eels and the other species examined.

It must be noted that some mortality occurred among eels in the same tanks so it is likely that the husbandry conditions for the eels were not optimal. Although all individuals used in the study appeared to be in good health, the serum activity may be

different to that which would be recorded in wild eels exposed to natural outbreaks of EUS.

The poor inhibition by most of the immune factors of tilapia measured in the present study is remarkable in the light of their EUS-resistance. Tilapia macrophages were unable to inhibit fungal growth, unlike those of silver barbs and gouramis, and there was no inhibition parameter in which tilapia macrophages or serum exceeded that of any other species. The effects of serum were particularly ambiguous as it could only inhibit germination at high temperature, but could only inhibit growth at low temperature.

Tilapia respiratory burst was at least as strong as that of susceptible species in all macrophage treatments. Wolf & Smith (1999) found that the granuloma response of hybrid tilapia *O. niloticus* x *O. mossambicus* x *O. aureus* was unusually effective against mycobacteriosis, suggesting that there are mechanisms available to tilapia macrophages which are not present in other species. However, such mechanisms may only be implied by the present study.

The relationships between silver barb and gourami respiratory burst and inhibition of germination indicate that the respiratory burst may be important in preventing the early stages of infection, though the weakness of the relationships suggest that it is not the only mechanism involved. Also, the efficacy of the respiratory burst in this context must be questioned, as neither species is resistant. The lack of a relationship between superoxide production and inhibition of growth in silver barbs suggests either that macrophages divert their resources to a different mechanism of inhibition, or that the germlings are actively interfering with the macrophages. The fact that the respiratory burst of inoculated macrophages is consistently lower than that of control macrophages similarly indicates one of these effects.

The negative regression of gourami macrophage superoxide production and inhibition of growth further suggests a change of mechanism. The macrophages of both silver barbs and gouramis are able to inhibit growth, which argues against the presence of inhibitory factors produced by the germlings. However, both species are susceptible and as the germlings in this study were only one day old, they may not yet have produced such factors in sufficient quantity to produce a significant effect. Virulence factors that inhibit superoxide production have not previously been observed in oomycete animal pathogens, though they are used by bacterial fish pathogens such as *Yersinia ruckeri* (Stave *et al.* 1987) and oomycete plant pathogens such as *P. infestans* (Doke 1983).

The only relationship between tilapia respiratory burst and inhibition was with PMA-stimulated macrophages, which probably does not indicate any relationship between respiratory burst and inhibition in a fish infected normally. It is more likely that the regression arises from the fact that both respiratory burst in the presence of PMA and inhibition of germination are both indicative of generally healthy macrophages.

The strong relationship between respiratory burst and inhibition of growth by low temperature snakehead macrophages suggests that the respiratory burst may be a major mechanism of defence in that species. However, inhibition of growth by snakehead macrophages was not significant, while snakeheads were the only species in which the respiratory burst was significantly inhibited in the presence of *A. invadans*. If the respiratory burst is as important to snakeheads as these data indicate, the high susceptibility of snakeheads to EUS may derive from the ability of *A. invadans* to inhibit it. Such strong relationships between respiratory burst and inhibition of growth

are not present in other species, implying the existence of other mechanisms that may not be available to snakeheads.

The respiratory burst is not the only antimicrobial mechanism available to macrophages. Nitric oxide has often been associated with fish granulocytes, and is involved in the killing of bacteria such as *Aeromonas hydrophila* (Yin *et al.* 1997). Human granulocytes secrete lysozyme to attack the hyphae of *Candida albicans* (Diamond *et al.* 1978), and lysozyme has been associated with fish granulocytes (Murray & Fletcher 1976). The role of such mechanisms in defence against hyphal fish pathogens such as *A. invadans* remains to be elucidated.

The ability of serum to inhibit germination was reduced by heating in all cases. This indicates that complement was responsible for the inhibitory activity as few other serum factors are inactivated at 55°C. If so, the role of complement appears to be important, as most sera completely lost their ability to inhibit germination after heating. Complement did not appear to be involved in the inhibition of fungal growth as silver barb and gourami serum did not lose growth-inhibiting activity after heating and unheated tilapia and snakehead serum did not inhibit growth. It is not clear whether complement was activated directly by the alternative pathway, or by the antibody-mediated classical pathway as snakeheads, gouramis and tilapia all had anti-*A. invadans* antibodies.

Only the high temperature snakehead serum inhibited germination after heating, although less effectively than unheated serum. This is probably related to the high anti-*A. invadans* antibody concentrations found in that group. The Suphanburi snakehead population suffered high mortalities when EUS first occurred in Thailand (Tonguthai 1985), and subsequent outbreaks were less severe, which may indicate the development of resistance (Chinabut 1998, Chapter 7). In recent years, Suphanburi snakeheads have

been widely used for experimental challenges (Chinabut *et al.* 1995, Thompson *et al.* 1997, Chapter 6), which indicates that the resistance conferred by the antibody response is far from complete. The inhibitory activity of snakehead serum in this study was restricted to germination, which suggests that, like complement, its importance is in preventing infection rather than controlling it once it has begun.

Superoxide production was higher at low temperature in all species, though only significantly in the case of snakeheads and control tilapia macrophages. Higher macrophage activity at low temperature has also been reported in tench *Tinca tinca* (Collazos *et al.* 1994) and common carp (Le Morvan *et al.* 1997), raising the possibility that the respiratory burst may be particularly important at temperatures where other mechanisms are suppressed. The apparent ability of *A. invadans* to withstand, and possibly inhibit, superoxide production, may explain the higher prevalence of EUS at relatively low temperatures where the respiratory burst is most important.

Macrophage adherence to plastic was used to separate macrophages from other cell types (Secombes 1990), and it was necessary to evaluate macrophage adherence to ensure that differences in inhibitory activity or respiratory burst were not simply a function of the number of macrophages present. Differences were found between the numbers of macrophages that adhered between species and between temperature groups. When macrophages were prepared for the fungicidal assays, it is unlikely that all cells identified as macrophages were actually macrophages and it is likely that the proportion of macrophages present at preparation varies between species. The number of macrophages recovered varied between species, but bore no relationship to the ability of macrophages to inhibit *A. invadans* or produce superoxide. Consequently, it is unlikely that comparisons of recovered macrophages between species are meaningful in terms of comparing responses to EUS.

Within species, it is unclear whether adherence itself is an indicator of any factor of the immune status of the fish. It has been suggested that high adherence of the macrophages of mammals (Kwak *et al.* 1998) and amphibians (Crumlish 1999) to plastic indicates a high activation state. Other measures of activation such as respiratory burst and phagocytosis are often used as indicators of the immune status of fish (Secombes 1990), but it is unclear whether the adherence of macrophages to plastic may be used as such a measure.

Within species, more macrophages were recovered at high temperature in the case of susceptible silver barbs and snakeheads, but there was no difference in the case of tilapia. If macrophage adherence indicates activation state, this data suggests that tilapia are less susceptible to the immunosuppressive effects of low temperature than susceptible species. Although the respiratory burst data suggested that snakehead macrophages produce more superoxide at low temperature, this data was collected only from the adhered, and presumably activated, macrophages. It is possible that the improvement in immune function among activated macrophages at low temperature may be offset if only small numbers of macrophages are actually activated at any one time.

Immunosuppression at low temperatures has been suggested as a likely mechanism for the seasonality of EUS. Studies *in vivo* have associated the resistance of otherwise susceptible species such as snakehead at high temperatures with the rapid development of granulomata (Chinabut *et al.* 1995). Bly & Clem (1992) suggested that granulomata form more slowly at low temperature due to the suppression of the T cells that control granulocyte activity by the release of cytokines, and Bly *et al.* (1992) suggested that T cell suppression may render channel catfish vulnerable to winter saprolegniasis (Section 1.4.3). If adherence does indicate the macrophage activation

state, the reduced adherence at low temperatures may indicate a similar effect in susceptible snakeheads and silver barbs that is absent in resistant tilapia.

Snakeheads, gourami and tilapia all had anti-*A. invadans* antibodies, although serum was not inhibitory in every case. Snakehead and tilapia both showed improved antibody production at high temperature. Carlson *et al* (1995) found that the antibody response of striped bass *Morone saxatilis* was enhanced at high temperatures, while macrophage activity was attenuated. If the antibody response is protective in snakeheads, seasonal immunosuppression may explain why successive outbreaks within the same area become more restricted to the colder months (Tonguthai 1985, Chinabut 1998).

5.5 Summary

- Macrophages of EUS-susceptible species, especially the highly susceptible snakehead, use the respiratory burst to inhibit the germination of *A. invadans* at low, EUS-permissive temperatures.
- *A. invadans* may be able to counter the macrophages by reducing their respiratory burst, especially those of snakeheads.
- Reduced macrophage adherence at low temperature may indicate low activation states in susceptible silver barbs and snakeheads.
- Tilapia macrophage adherence was unaffected at low temperature, suggesting less attenuation of activation than in susceptible species.
- Serum of susceptible species inhibits *A. invadans*, but serum of tilapia does not, suggesting that serum factors are important but do not provide complete protection.
- Inhibition of germination by serum is prevented by heating the serum, indicating that one of the serum factors involved is complement.

- Anti-*A. invadans* antibodies were found in all species in which they were looked for.
- The anti-*A. invadans* antibody concentrations were reduced at low temperatures in snakeheads and tilapia, suggesting that protection conferred by antibodies is restricted to warmer seasons.
- Other serum factors that were not identified are involved in the inhibition of *A. invadans*, especially in the inhibition of growth.

6 Immunostimulation of Fish Against *Aphanomyces invadans**

6.1 Introduction

At present, the control of EUS is based on water treatment and management strategies (Lilley *et al.* 1998). Antibiotics have also proved useful in controlling EUS, but the danger of promoting the spread of antibiotic resistance makes their use inadvisable (Section 1.1.6).

Since the discovery that adjuvants used in the application of vaccines confer considerable resistance in themselves (Olivier *et al.* 1985), a wide range of products have been tested for immunostimulatory effects in fish (Sakai 1999). Early studies concentrated on killed pathogens such as *Aeromonas salmonicida* (Chung & Secombes 1987), but it was also found that structural compounds commonly found in microbes, such as glucans (Yano *et al.* 1989) and lipopolysaccharides (Hine & Wain 1988), had similar effects.

As research on immunostimulants expanded, many apparently unrelated substances were found to enhance the immune response when added to feed. Candidates were as diverse as the anthelmintic levamisole (Kajita *et al.* 1990), bovine lactoferrin (Sakai *et al.* 1993) and chicken egg fermentation products (Yoshida *et al.* 1993).

Vadstein (1997) suggested that immunostimulation may be useful in situations where vaccination is inappropriate, citing hatcheries where the main health risks are opportunistic pathogens and the immune systems of the fish have not developed

* The studies described in this chapter have been published as follows:

Miles, D.J.C., Polchana, J., Lilley, J.H., Kanchanakhan, S., Thompson, K.D. & Adams, A. (2001). Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. *Aquaculture* 195:1-15.

sufficiently to develop adaptive immunity. The situation on many Asian farms may be added to his list, where it is unlikely to be financially or practically possible to apply vaccines.

Striped snakehead *Channa striata* farms in Thailand are typical of such systems. Wild caught fingerlings are placed in grow-out ponds, and harvested after approximately 10 months. The fish are not collected at any point between seeding and harvest, so there is little opportunity to apply a vaccine by injection or immersion. On the other hand, most of their feed is provided in the form of trash fish. The fish is usually mechanically minced and mixed with rice bran, and an oral immunostimulant may be introduced during the process.

Many methods of testing immunostimulants have been used. Substances may be injected or mixed with feed, and the results may be tested by examination of the immune parameters of the fish or challenge with an experimental pathogen (Sakai 1999). However, while injection followed by examination of immune parameters is the simplest method of screening, it may not replicate the effect that the immunostimulant would have in fish exposed to natural outbreaks. Farm trials, on the other hand, are expensive and may lead to ambiguous results.

Galeotti (1998) suggested that *in vitro* screening methods should be used to elucidate the mechanisms of immunostimulation, then *in vivo* methods should be used to establish whether the benefits occur in live fish. This study followed such an approach by following preliminary screening of a range of putative immunostimulants by injection with examination of immune parameters *in vitro*. The most effective substances were selected for oral administration to fish, which were challenged with *A. invadans*.

6.2 Methods

6.2.1 Studies *in vitro*

Five substances were injected into fish to assess their immunostimulatory properties.

6.2.1.1 Experimental fish

Striped snakeheads weighing 66.7g (sd \pm 17.7) were acquired from pond farms in Suphanburi province, Thailand and placed in 50l opaque plastic tanks with a through-flow system at a density of 5 fish tank⁻¹. They were acclimatised at 21°C \pm 1°C for 14d before the trial to simulate the temperature at which EUS may be expected to occur (Tonguthai 1985, Chinabut *et al.* 1995). Fish were fed twice daily to satiation with CP brand no. 9910 pellets containing at least 30% protein.

6.2.1.2 Application of immunostimulants.

Table 6.1 lists the immunostimulants used, all of which were in the form of insoluble powders. All were provided by AVL.

Table 6.1. Active ingredients of putative immunostimulants used for *in vitro* trial.

Product	Active ingredient [*]
Salar-bec	300g kg ⁻¹ vitamin C, 150g kg ⁻¹ vitamin E, trace quantities of vitamins B ₁ , B ₂ , B ₆ , B ₁₂
Ergosan	0.002% unspecified plant extract, 1% alginic acid from <i>Laminaria digitata</i> , 98.998% algal based carrier.
Betamak C85	Brewers' yeast containing 32% β 1,3 & 1,6 Glucans, 30% unspecified mannan.
Lysoforte	Lysophospholipids
Oro glo layer dry	Yellow xanthophylls derived from marigold <i>Tagetes erecta</i> , principally lutein with significant amounts of zeaxanthin.

^{*}Information courtesy of AVL and Elorisan GmbH-Biostimulatoren, Deggendorf, Germany.

For application, the immunostimulants were suspended at 5mg ml⁻¹ in autoclaved PBS. Fish were lightly anaesthetised with benzocaine solution until movement ceased. Each fish was injected intraperitoneally with 100µl of the immunostimulant suspension with a 21g hypodermic needle (Kajita *et al.* 1990, Robertsen *et al.* 1990).

One group of five fish was used per treatment and a control group was injected with autoclaved PBS only.

6.2.1.3 Assessment of effect of immunostimulant

Fish were killed and weighed, and macrophage and serum fungicidal assays were prepared (Section 5.2.5) using *A. invadans* isolate NJM9701. Assays of heated serum were not used. All replicates of any one treatment had the same control. Macrophage counts were omitted due to the preliminary nature of the trial.

6.2.1.4 Statistical analysis

Inhibition of germination and growth by macrophages and sera of different treatment groups were compared by nested ANOVA's followed by Tukey multiple comparisons of the indices of inhibition (Section 5.2.9).

6.2.2 Challenge *in vivo*

The effects of Salar-bec and Ergosan on the ability of striped snakeheads and silver barbs to withstand challenge with *A. invadans* were assessed.

6.2.2.1 Experimental fish

Striped snakeheads were acquired from the same source as in the study *in vitro* (Section 6.2.1.1). Silver barbs were acquired from NAGRI. Fish were placed in 150l glass tanks at a density of 40 fish tank⁻¹. Two tanks of each species were allocated as treatments

and two as controls for each immunostimulant. Water quality was maintained by frequent water changes. All fish were fed twice daily to satiation on CP brand no. 9910 pellets.

Fish were acclimatised at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 14d prior to the experiment.

6.2.2.2 Application of immunostimulant

The most successful immunostimulants from the study *in vitro*, Salar-bec and Ergosan, were selected for the *in vivo* trial. Immunostimulant was added to feed pellets at 2g kg^{-1} and mixed, then 10ml vegetable oil was added to the mixture to attach the immunostimulant to the pellets. Control feed was prepared by adding oil only.

After acclimatisation, fish were placed on experimental diets for 14d prior to challenge with *A. invadans*.

6.2.2.3 Challenge

A zoospore suspension of $100\text{ zoospores ml}^{-1}$ of isolate B99C was assessed and prepared with a modified Neubauer haemocytometer. A $100\mu\text{l}$ dose of the suspension was injected intramuscularly into the right of the leading edge of the dorsal fin of each fish.

6.2.2.4 Sampling protocol

Three fish were sampled from each tank at 5d intervals by random scoop with a net from the day of the challenge to day 40. Day 35 samples were omitted from the Salar-bec trial as there were not enough fish left in several tanks to allow for sampling on both days 35 and 40.

Each fish was weighed and the injection site excised and fixed in 10% neutral buffered formalin for histological examination. Blood samples were taken from the caudal blood vessels and serum collected for assessment of RAC.

Incidental mortalities were also recorded, though histopathology was not carried out on these fish, as there was time for decomposition to begin.

6.2.2.5 Histopathology

All samples were prepared and stained with GMS-H&E (Section 2.2).

The presence or absence of invasive fungal hyphae was recorded. The cellular immune response was classified either as acute inflammation, with infiltration of granulocytes into the region of infection but little or no organisation, or chronic inflammation where distinct granulomata could be observed enclosing hyphae (Fig 6.1). The area of each type of response in each slide was measured with a grid graticule. An index of inflammation was calculated by dividing the cross-sectional area of acute inflammation by the cross-sectional area of chronic inflammation.

6.2.2.6 Assessment of RAC

The RAC was quantified by ELISA, using MAb 7D2 (Section 5.2.8). No assessment was possible for the silver barbs as there was no available MAb.

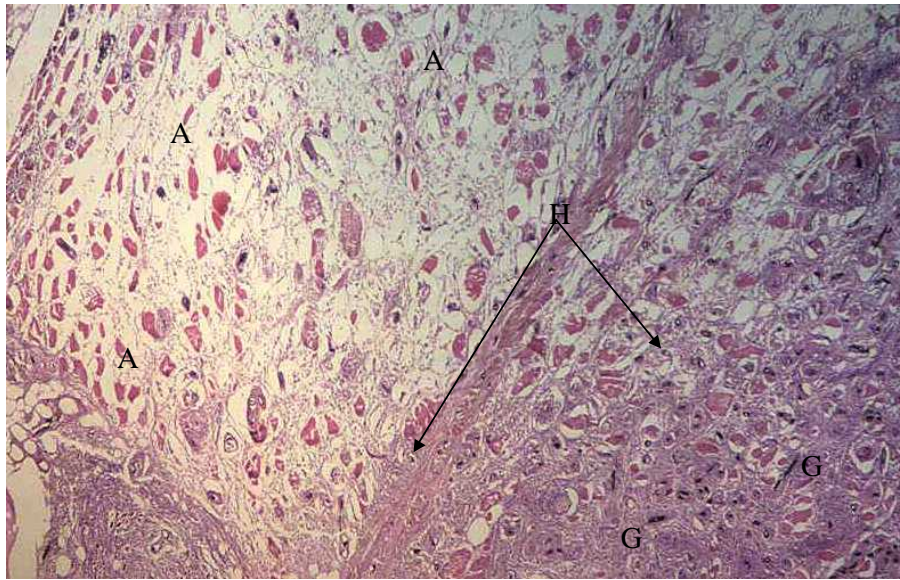
6.2.2.7 Statistical analysis

The weights of fish of the same species treated with the same immunostimulant and the equivalent control fish were compared by 1-way ANOVA.

Incidence of EUS in control and treatment tanks was compared by Yates corrected λ^2 analysis.

Differences in mortality between treated fish and their controls were assessed by Kaplan-Meier survival analysis.

a/



b/

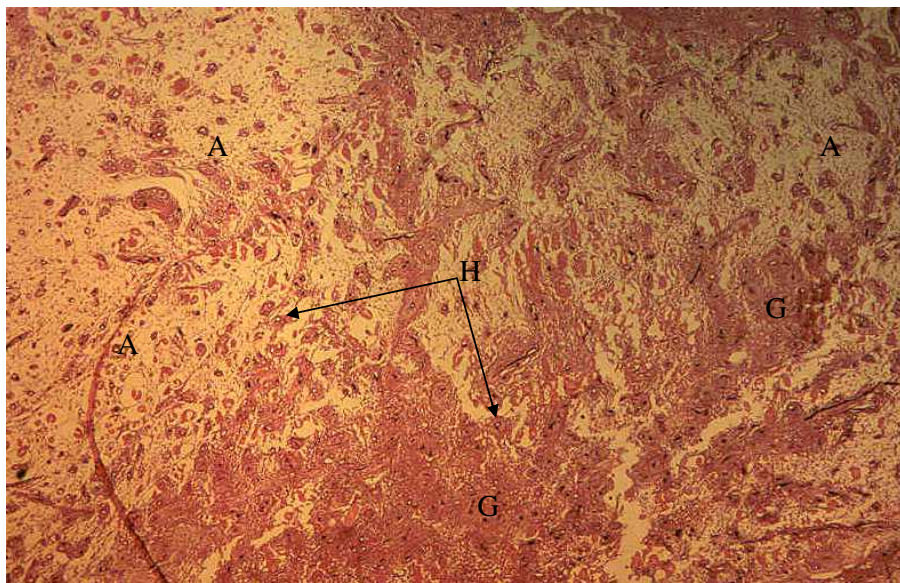


Fig. 6.1. Muscle sections of a/ striped snakehead and b/ silver barb 20d after injection with *A. invadans* zoospores, illustrating hyphae (H), granulomata (G) and acute inflammation (A). Stained with H&E and photographed at 40x magnification.

The areas and ratios of the different types of inflammatory response were plotted against fish weight and time after challenge. If a relationship was evident, its strength was assessed by regression analysis and the significance assessed by F-test. If there was no relationship, the histopathology of control and treatment fish was compared by t-test. If a relationship was present, control and treatment fish were compared by ANCOVA. A similar approach was used to compare the RACs of treated and control fish.

6.3 Results

6.3.1 Studies *in vitro*

Five substances were injected into striped snakeheads to assess their immunostimulatory properties.

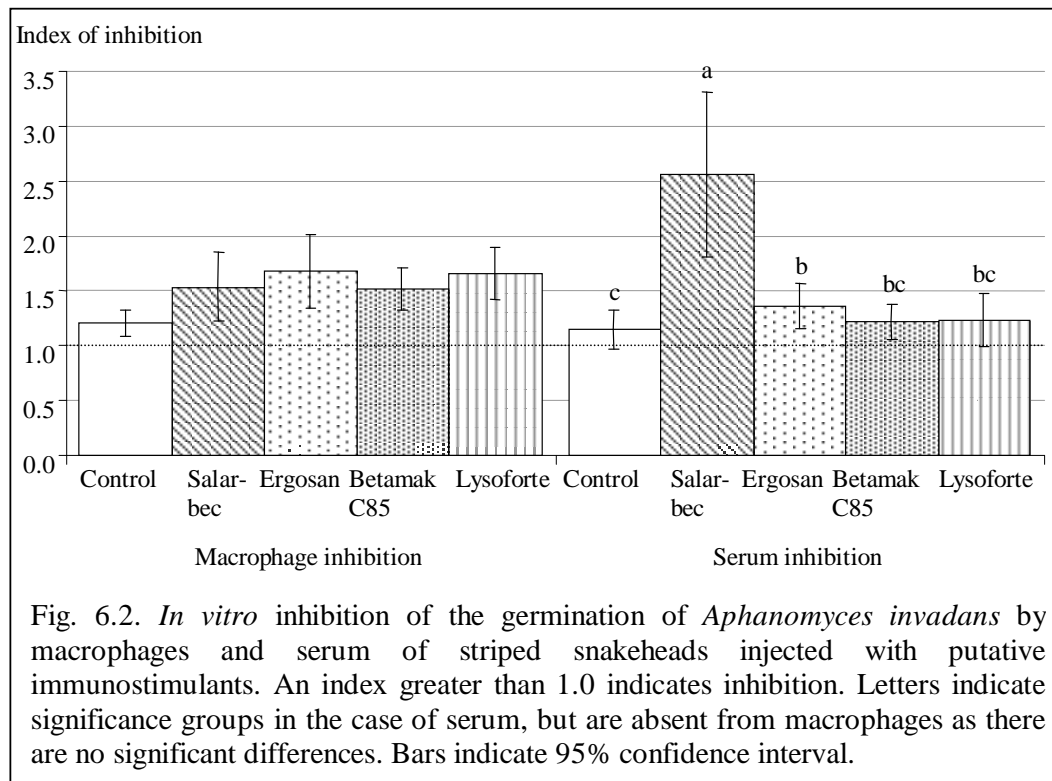
6.3.1.1 Mortality

Of the five fish injected in each treatment group, one fish died before sampling in every group including the control group, except in the case of fish injected with Oro Glo Layer Dry where three of the five fish died. No further studies were carried out on Oro Glo Layer Dry, as there were insufficient survivors to make up a sample group.

6.3.1.2 Inhibition of germination

There were no differences in the inhibitory activity of macrophages of the different groups ($F = 1.12$, $df = 4$, $p > 0.05$), though there were differences in serum activity ($F = 5.98$, $df = 4$, $p < 0.01$).

Sera of fish injected with Salar-bec were more inhibitory than that of any other group ($p < 0.001$). Sera of fish injected with Ergosan were more inhibitory than that of control fish ($p < 0.01$), though they were not significantly different to that of fish



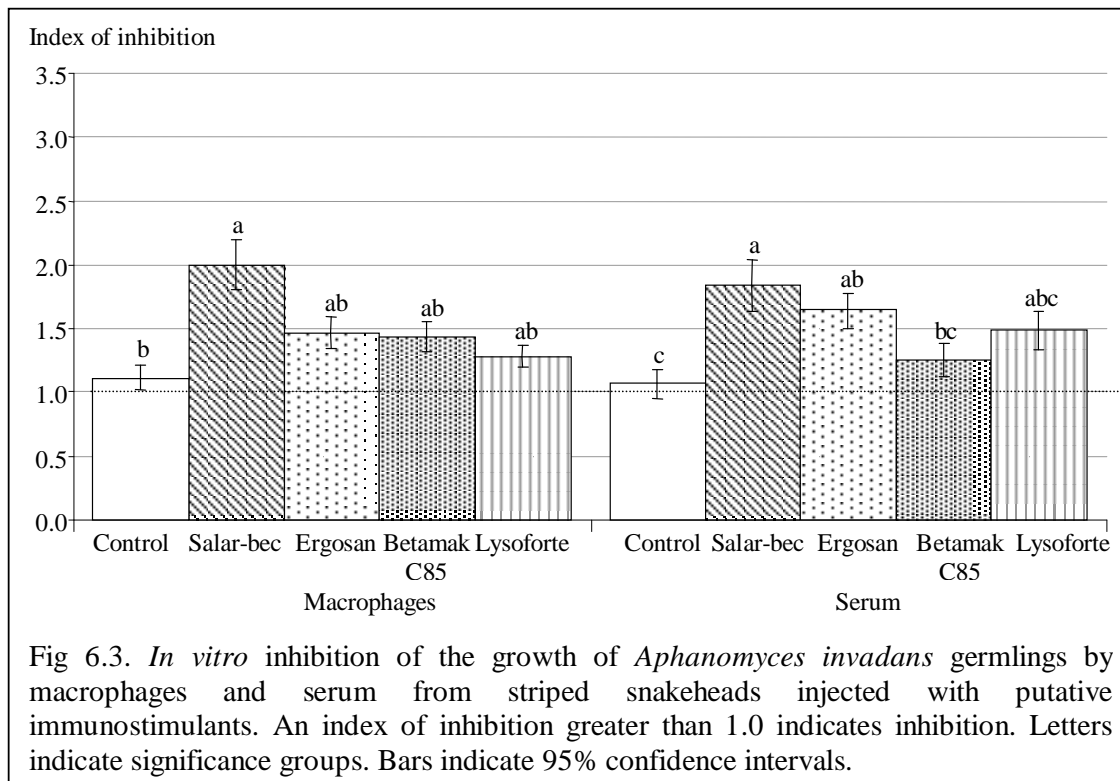
injected with Betamak C85 or Lysoforte ($p > 0.05$). There were no other significant differences (Fig. 6.2).

6.3.1.3 Inhibition of growth

There were differences in the inhibitory activity of both macrophages ($F = 17.59$, $df = 4$, $p < 0.0005$) and serum ($F = 12.52$, $df = 4$, $p < 0.0005$).

Macrophages of fish injected with Salar-bec were more inhibitory than those of control fish ($p < 0.05$), though there were no other differences and there was no variation between individuals ($p > 0.05$).

Sera of fish injected with Salar-bec were more inhibitory than sera of control fish ($p < 0.005$) or fish injected with Betamak C85 ($p < 0.05$), though not more than sera of fish injected with Lysoforte or Ergosan ($p > 0.05$). Sera of fish injected with Ergosan were more inhibitory than control fish sera ($p < 0.005$), but not different to sera of fish injected with Lysoforte or Betamak C85 ($p > 0.05$) (Fig. 6.3).



6.3.2 Challenge *in vivo*

Striped snakeheads and silver barbs were fed on diets supplemented with Salar-bec or Ergosan, and challenged with *A. invadans* by intramuscular injection.

6.3.2.1 Weights

The weights of all fish sampled were compared (Table 6.2). No differences were found between tanks of the same species involved in the trial of either immunostimulant ($p > 0.05$).

6.3.2.2 Incidence

Incidence of EUS, as determined by histopathology, was recorded among the fish randomly sampled in the Salar-bec trial (Fig. 6.4). There were no differences between the treatment and control fish in the case of either snakeheads ($\lambda^2 = 0.03$, $df = 1$, $p > 0.05$) or barbs ($\lambda^2 = 0.59$, $df = 1$, $p > 0.05$).

Table 6.2. Weights of sampled fish from the *in vivo* immunostimulant trial.

	Species	Mean weight (g)	Standard deviation
Salar-bec	Striped snakehead	46.07	17.10
	Silver barb	17.84	7.37
Ergosan	Striped snakehead	48.52	19.81
	Silver barb	12.24	15.62

The aquarium in which the Ergosan trial was carried out suffered an air-conditioning failure on the day after the challenge and temperatures rose to over 35°C for approximately 24h. Probably because of the high temperatures, prevalence among sampled fish was very low and only two barbs (4%) from both the treatment and control groups had EUS. No sampled snakeheads had EUS.

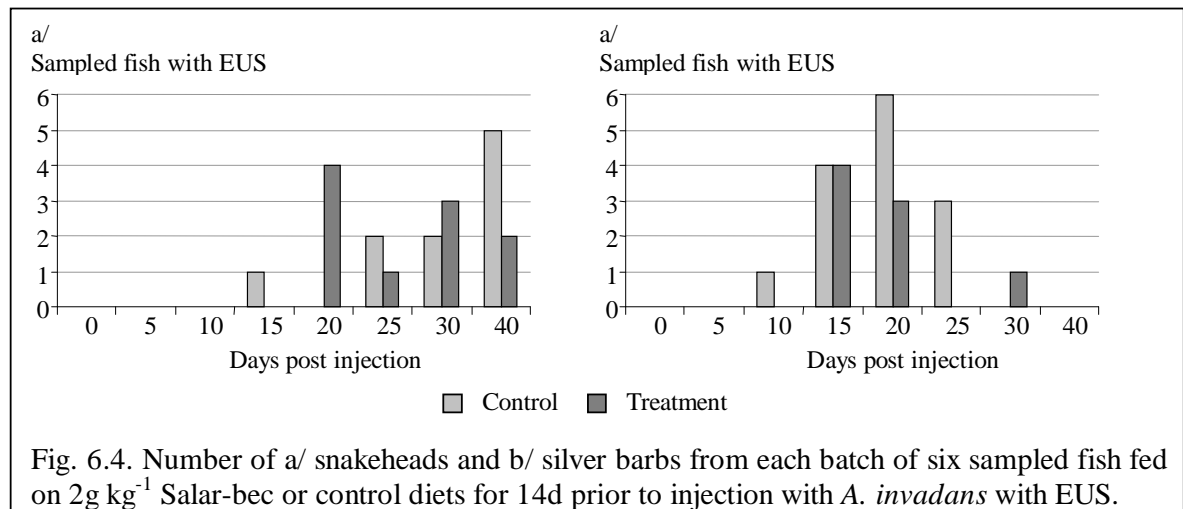


Fig. 6.4. Number of a/ snakeheads and b/ silver barbs from each batch of six sampled fish fed on 2g kg⁻¹ Salar-bec or control diets for 14d prior to injection with *A. invadans* with EUS.

6.3.2.3 Mortality

Mortalities among unsampled fish were recorded.

In the Salar-bec trial, one barb treatment tank suffered massive mortality on day seven due to an aeration failure, and that tank was removed from all subsequent

analysis. Aeration failure caused a less severe mortality in the second Salar-bec barb treatment tank on day 28 and those deaths were censored from the analysis. That tank had a possible maximum mortality of seven, while all other tanks in the Salar-bec trial had a possible maximum of 16.

After these reservations, Salar-bec improved the relative percent survival (RPS) (Ellis 1988a) of barbs by 23% (Kaplan-Meier statistic = 10.26, df = 1, $p < 0.005$) and that of snakeheads by 59% (Kaplan-Meier statistic = 15.63, df = 1, $p < 0.0005$).

There was a possible maximum mortality of 13 in all tanks in the Ergosan trial due to the extra day of sampling. Ergosan improved the RPS of snakeheads by 60% (Kaplan-Meier statistic = 5.23, df = 1, $p < 0.05$), but only two barbs (8%) died from the control and two (8%) from the treatment groups so no analysis was possible (Fig. 6.5).

6.3.2.4 Inflammatory response

Covariance of time after injection and fish weight on the area of acute inflammation, area of granulomatous response, total inflamed area and index of inflammation were compared by regression analysis with F-test. No results were forthcoming from the fish treated with Ergosan due to the low numbers infected. Salar-bec treated snakeheads did not vary from the controls in terms of either the area of acute ($F = 0.08$, df = 1, $p > 0.05$) or total inflammation ($t = 1.14$, df = 19, $p > 0.05$). Nor was there any relationship between time from injection and either variable. The total area of chronic inflammation regressed logarithmically with time in treated snakeheads, though statistical significance was equivocal ($r^2 = 0.32$, $F = 4.74$, df = 1, $p = 0.055$) (Fig. 6.6). A stronger logarithmic regression was evident between the index of inflammation and time ($r^2 = 0.46$, $F = 6.72$, df = 1, $p < 0.05$) (Fig. 6.7). Neither relationship was found in the case of control snakeheads ($p > 0.05$) and there was no relationship between fish size and either variable.

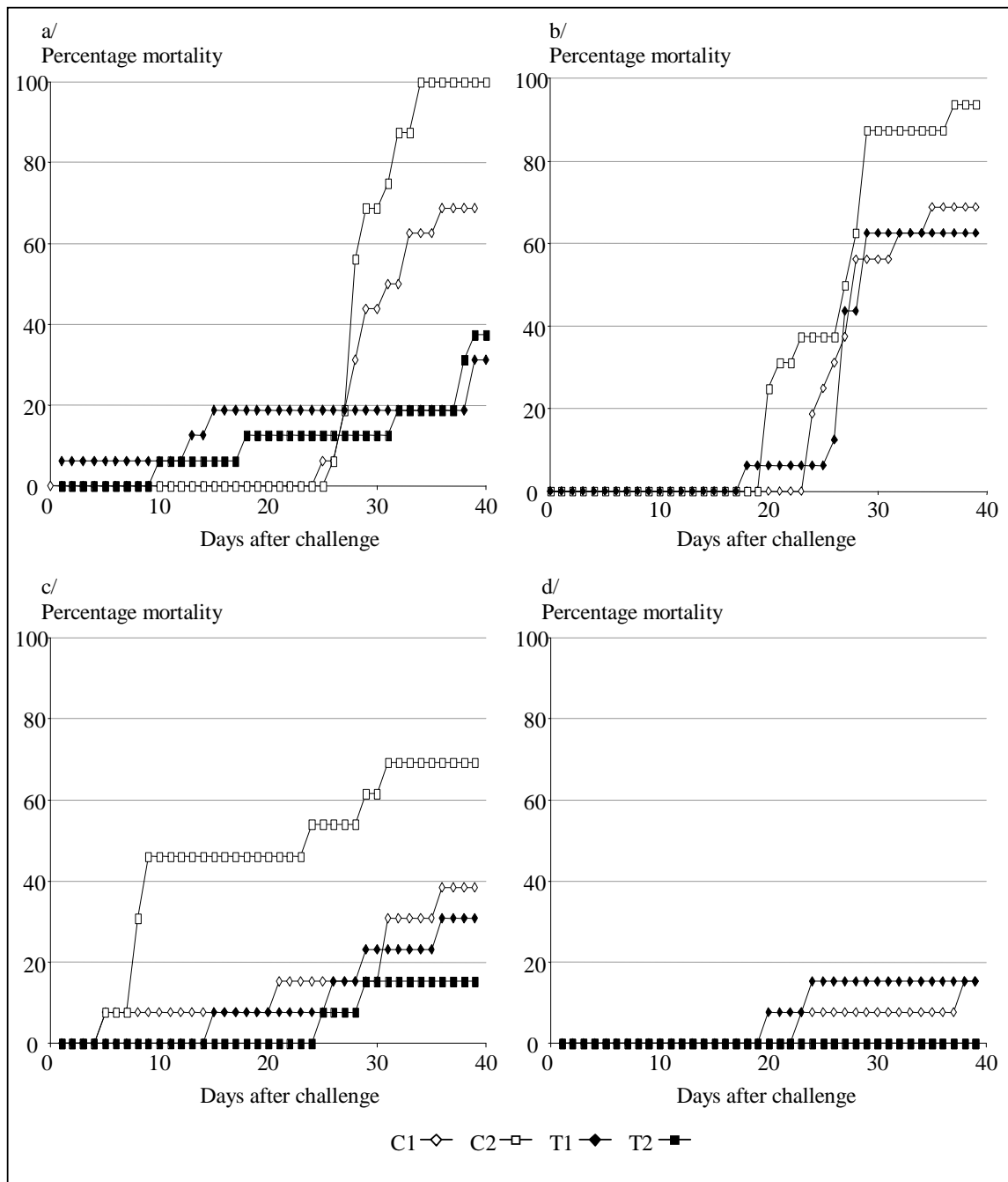
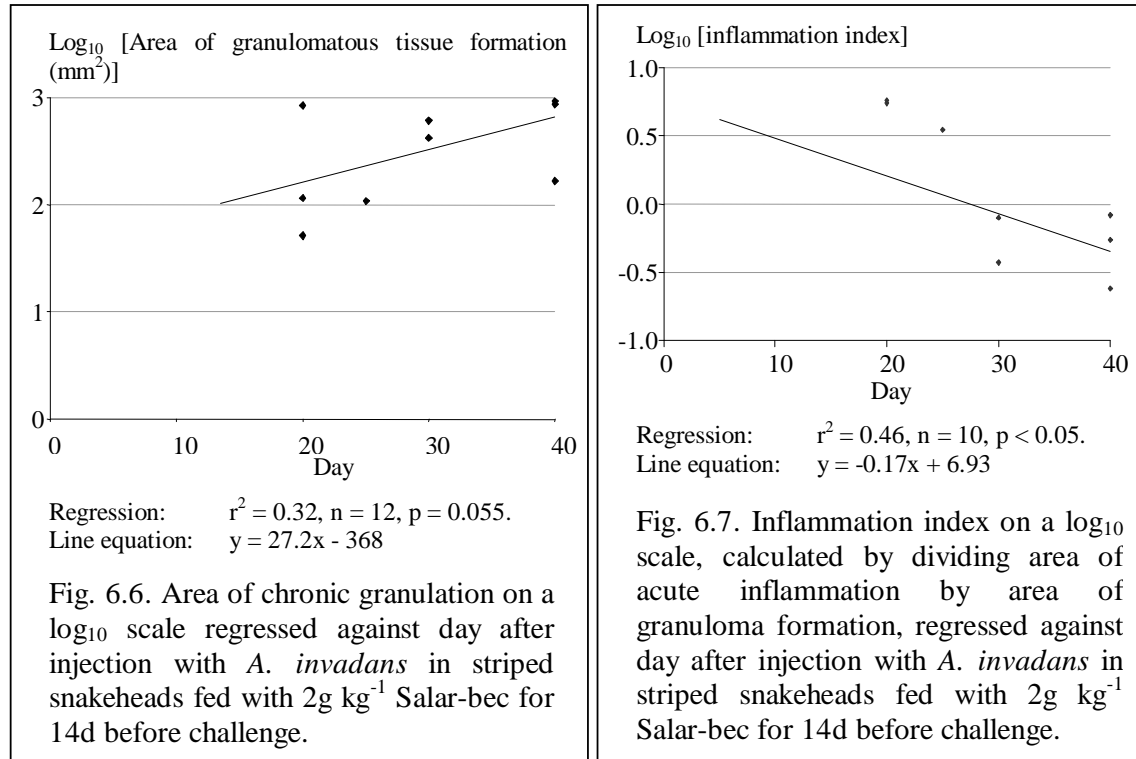


Fig. 6.5. Cumulative percentage mortality after injection with *Aphanomyces invadans* cysts of a/ striped snakehead fed on Salar-bec supplemented feed, b/ silver barbs fed on Salar-bec supplemented feed, c/ striped snakeheads fed on Ergosan supplemented feed and d/ silver barbs fed on Ergosan supplemented feed. Results from control tanks (C1, C2) and treatment tanks (T1, T2) are separated. Mortalities due to aeration failure on d28 in T1 of part b were disregarded.

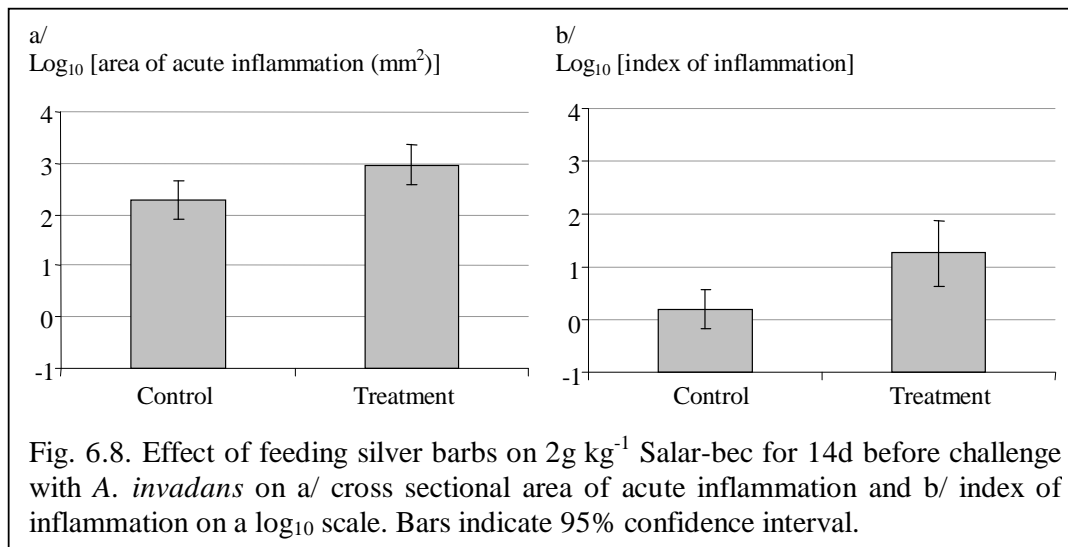
Treated snakeheads had more chronic inflammation by area than control snakeheads ($F = 4.78$, $df = 1$, $p < 0.05$) (Fig. 6.6), but there was no difference between the indices of inflammation ($F = 1.93$, $df = 1$, $p > 0.05$) (Fig. 6.7).



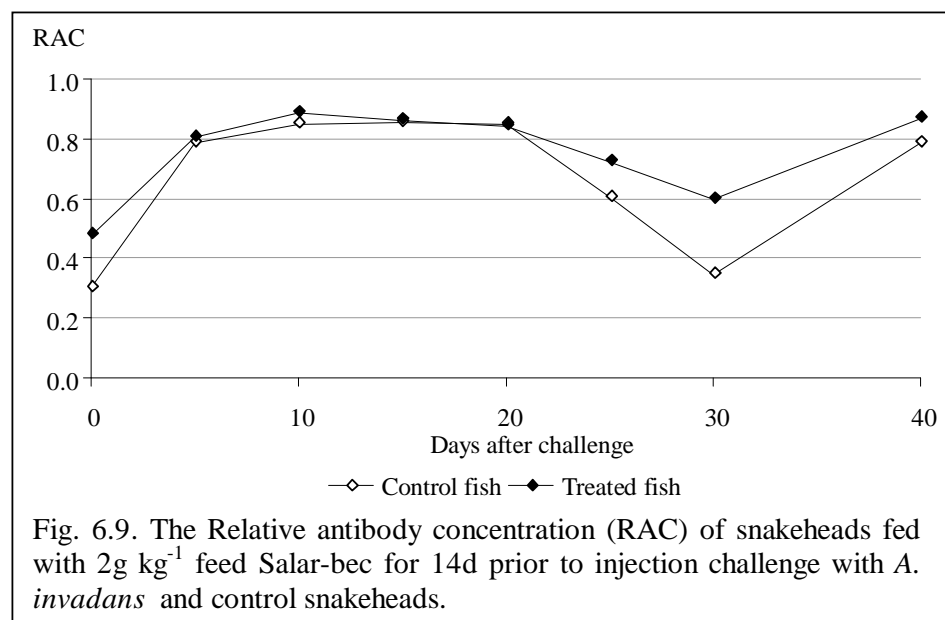
There was no relationship of any aspect of the inflammatory response of silver barbs with weight or time. There was no difference in the total inflamed area ($t = 2.27$, $df = 20$, $p > 0.05$) or area of chronic inflammation ($t = 0.92$, $df = 12$, $p > 0.05$) between treated and control fish. However, a greater proportion of the inflammatory response of treated barbs was acute, which was reflected in both the absolute area of acute ($t = 2.42$, $df = 20$, $p < 0.05$) and the index of inflammation ($F = 9.16$, $df = 1$, $p < 0.05$) (Fig. 6.8).

6.3.2.5 RAC

The RAC of anti-*A. invadans* antibodies was assessed in all sampled snakeheads. There was no relationship between RAC and time in any case.



Snakeheads treated with Salar-bec had a consistently higher RAC than control snakeheads ($t = 2.45$, $df = 77$, $p < 0.05$) (Fig. 6.9). There was no difference between snakeheads treated with Ergosan and controls ($t = 0.46$, $df = 105$, $p > 0.05$).



6.4 Discussion

Five substances were evaluated for their ability to stimulate the immune response of striped snakehead to *A. invadans*. Striped snakeheads were selected as they are a widely

used model for EUS studies (Cruz-Lacierda & Shariff 1994, Chinabut *et al.* 1995, Thompson *et al.* 1997) as well as being commercially important.

The most effective immunostimulants, Salar-bec and Ergosan, were selected for a challenge experiment. The challenge was carried out on striped snakehead, and also on silver barbs as it was desirable to establish whether any effects occurred in species other than snakeheads. Silver barbs are cultured in many Asian countries in systems that are often badly affected by EUS (Tonguthai 1985, Roberts *et al.* 1989, McAndrew in press). Lysoforte, Betamak C85 and Oro Glo Layer Dry were not pursued as they were less effective.

The performance of Lysoforte-injected snakeheads was generally poor, although it was consistently better than the controls. The lysophospholipids in Lysoforte are used as biosurfactants to improve feed conversion in mammal and poultry farming (Schwarzer & Adams 1996), though their effect on the immune system has not been studied in any animal.

Snakeheads injected with Betamak C85 showed little or no difference to controls by any parameter measured. Many studies have shown yeast extracts containing β glucans and mannans similar to Betamak C85 to have immunostimulatory properties (Sakai 1999), and they are widely used in salmonid farming (Raa 1996). Few studies have examined their effect on the immune response to eukaryote pathogens, though a similar extract gave sand whiting *Sillago ciliata* some protection against *A. invadans* (Catap & Munday 1998b). Robertsen *et al* (1994) suggested that β glucans are most effective against opportunistic pathogens, which may explain their lack of efficacy against the obligate pathogen, *A. invadans*. They also report that the effect of β glucans is dose dependant as low or high doses afford no improvement in the immune system,

and very high doses may be inhibitory. Such inhibition may have occurred here as the appropriate dose rate for striped snakeheads is unknown and may have been exceeded.

Oro Glo Layer Dry, a preparation of yellow xanthophylls used as a pigment enhancer in poultry farming, was rejected because it caused a higher mortality among snakeheads injected with it than any other treatment or the control. The immunomodulatory properties of xanthophylls are unclear, though other carotenoids such as β carotene are vitamin A precursors, and show a range of immunostimulatory and antioxidant activity in mammals (Chew 1993).

Fish injected with Ergosan showed improvement in the ability of serum to inhibit both growth and germination *in vitro*. Such alginates were first used by the aquaculture industry as binders for pelleted feed, but immunomodulatory properties have been found in extracts from phytophagae such as *Laminaria digitata*, the source of the alginates in Ergosan (Dalmo *et al.* 1998, Gabrielson & Austreng 1998). Hall (1998) reported the immunostimulatory effects of Ergosan itself on Atlantic salmon.

Like most alginates, Ergosan contains polyuronic acids that 'act in combination with certain cations to enhance the transfer of oxygen to cell membranes. The [polyuronic acid] macromolecule attaches to the cell surface and acts there as a phase transfer catalyst' (Elorisan GmbH-Biostimulatoren, unpublished data). Dalmo *et al* (1998) attributed the immunomodulatory properties of a similar alginate, laminaran, to the fact that it contains β glucans. Ergosan also contains β glucans, but not in a form available to fish (Elorisan GmbH-Biostimulatoren, unpublished data).

The very low incidence of infection by *A. invadans* in the Ergosan trial makes it impossible to evaluate Ergosan as a potential prophylactic against EUS as mortalities

could not be confidently attributed to EUS. However, Ergosan did reduce the mortality of snakeheads when used as a feed supplement.

Salar-bec was the most effective immunostimulant in the preliminary study as the effects of macrophages and serum on growth and serum on germination were stronger in snakeheads injected with Salar-bec than any other treatment. Further, serum was more inhibitory of growth than in any treatment except Ergosan. Both snakeheads and silver barbs fed on diets supplemented with Salar-bec survived better when challenged with *A. invadans*.

Although incidence of infection was not reduced, the effect on mortality suggests that Salar-bec improved ability to control the spread of the mycelium. In both species, hyphae appeared later in treated fish of both species than in controls, suggesting that Salar-bec enhanced the ability of the fish to delay the proliferation of the infection. There was also evidence for faster granuloma development in snakeheads fed on Salar-bec. Chinabut *et al.* (1995) found that striped snakeheads injected with *A. invadans* at 26°C or above developed granulomata relatively quickly and recovered, while others at lower temperatures showed only acute inflammation and died. A rapid granuloma response has also been suggested as a characteristic of species resistant to EUS (Wada *et al.* 1996). It appears that Salar-bec induced an immune response in snakeheads that is typical of fish resistant to EUS.

No such effect on granuloma formation occurred in silver barbs, suggesting that most inhibition is mediated through the acute inflammatory response. The larger area of acute inflammation in treated barbs when compared to controls further supports such a possibility.

Snakeheads fed with Salar-bec had higher anti-*A. invadans* antibody concentrations than controls. Other studies (Chapter 5, Chapter 7) suggest that the

antibody response elicited in striped snakeheads may afford some protection, and it is possible that the higher antibody concentrations elicited by Salar-bec contributed to the ability of the fish to contain the infection.

Several studies have shown the value of orally administered vitamin supplements as immunostimulants, including vitamin C in channel catfish (Durve & Lovell 1982, Li & Lovell 1985), Atlantic salmon (Erdal *et al.* 1991, Hardie *et al.* 1991) and rainbow trout (Navarre & Halver 1989, Wahli *et al.* 1995). Other studies have shown the immunostimulatory properties of vitamin E in channel catfish (Wise *et al.* 1993) and turbot *Scophthalmus maximus* (Pulsford *et al.* 1995). Of particular interest is Durve and Lovell's (1982) observation that vitamin C improved the resistance of channel catfish to edwardsiellosis at low temperature, implying that it may be used to counter temperature mediated increases in susceptibility such as are commonly seen in EUS outbreaks.

No laboratory-based trial can exactly replicate the situation in a pond farm where fish are exposed to a wide range of environmental variation and a complex microbial biota. Similarly, injecting a pathogen into the fish does not replicate a natural outbreak. A further concern is that the fish in the trial were fed on pelleted feed, in which the vitamin content is likely to deteriorate rapidly during processing and storage (Soliman *et al.* 1987). Adding fresh vitamins may improve pelleted feed more dramatically than it would in the case of fresh trash fish, which is used on snakehead farms, where vitamin loss may be less severe.

A further weakness of this study is that as no histopathology was performed on fish that died in the course of the experiment as the tissue would have begun to decompose, so *A. invadans* itself was not positively identified as the cause of the mortality. However, it is far from clear whether fish killed in natural infections are

killed by *A. invadans* itself or by secondary infections entering through the lesions formed by *A. invadans* infection (Boonyaratpalin 1989). It is clear that there was a high incidence of EUS among live fish in the Salar-bec trial, so the situation was analogous to that which would be encountered when sampling an EUS-infected pond farm.

While some caution should be exercised in extrapolating the results of this study to the likely effect of immunostimulants on farms, it provides strong evidence that Salar-bec may be of use in controlling EUS subject to optimisation of dose regime and assessment in pond trials.

6.5 Summary

- Products that enhanced the immune system after intraperitoneal injection also enhanced immunity to *A. invadans* when mixed with feed.
- The xanthophyll preparation Oro Glo Layer Dry, the lysophospholipid Lysoforte, and the brewer's yeast preparation Betamak C85 showed little or no potential for enhancing resistance to EUS.
- Intraperitoneal injection of the vitamin supplement, Salar-bec, enhanced the anti-*A. invadans* activity of the macrophages and serum of striped snakeheads.
- Administering Salar-bec in the feed of striped snakeheads enhanced their antibody production, speeded up their granulomatous response to *A. invadans* and reduced their mortality after challenge with *A. invadans*, although it did not affect incidence.
- Administering Salar-bec in the feed of silver barbs enhanced their acute inflammatory response to *A. invadans* and reduced mortality after challenge with *A. invadans*, although it did not affect incidence.
- Intraperitoneal injection of the alginate, Ergosan, enhanced the anti-*A. invadans* activity of the serum of striped snakeheads.

- Administering Ergosan in the feed of striped snakeheads reduced the mortality of striped snakeheads challenged with *A. invadans*, although very little EUS occurred in the course of that trial.

7 Acquired Immunity of Wild Striped Snakehead *Channa striata* to *Aphanomyces invadans*

7.1 Introduction

The ability of fish to improve their immunity to a specific pathogen after exposure to it has been known for nearly 60 years (Duff 1942). The discovery that this immunity is mediated by antibodies similar to those produced by mammals was not made until some time later (Post 1966), and marked the beginning of research into the possibility of vaccinating fish.

Since then, it has been shown that antibodies produced by fish confer protection to a range of bacterial (Anderson & Nelson 1974, Antipa *et al.* 1980, Gudmundsdottir & Magnadottir 1997), parasitic (Speed & Pauley 1984, Whyte *et al.* 1990) and viral (Nishimura *et al.* 1985) diseases.

Antibody-mediated protection is well documented for a range of hyphal diseases of mammals including human aspergillosis (Schønheyder & Andersen 1983), the equine dermatophyte *Trichophyton equinum* (Pier & Zancanella 1993) and the equine oomycosis caused by *Pythium insidiosum* (Mendoza *et al.* 1992a, b). There are no reports of studies of antibody-mediated immunity to any hyphal pathogen of fish.

Previous studies on EUS have established the presence of antibodies that react to *A. invadans* in EUS-susceptible fish, particularly striped snakehead (Thompson *et al.* 1997, Chapter 5), and associated them with improved resistance to EUS (Chapter 5, Chapter 6). However, the only study on the response of fish to EUS to use fish that were not collected from an EUS-endemic region found that EUS-naïve rainbow trout

had anti-*A. invadans* antibodies (Thompson *et al.* 1999). The absence of anti-*A. invadans* antibodies in EUS-naïve fish of susceptible species has not been shown.

The acquisition of immunity may be implied by the decline in prevalence and severity of EUS in affected areas (Chinabut 1998, Section 1.1.4), but it has never been clearly shown that antibodies confer protection to EUS or are responsible for the decline.

Detection of pathogens in wild populations poses many problems, as the scale of a study powerful enough to conclude that a pathogen is absent from an environment is usually prohibitively large. Such detection is particularly important in the case of EUS as no method exists to locate *A. invadans* other than by histopathological examination of fish that are already infected. The danger of transmission of EUS from wild fish to pond or cage farms (McAndrew in press, Section 1.1.4) makes it important to establish whether it is present in the environment before it reaches the farms, allowing farmers to take appropriate control measures (Section 1.1.6).

Immunoprevalence monitoring involves tracing the geographical range of a pathogen by looking for antibodies in the host population, and has been used for a number of hyphal diseases of wild animals, such as penguin aspergillosis (Graczyk & Cockren 1995). A large scale study on wild salmonid populations led to the suggestion that immunoprevalence monitoring provided a reliable and manageable method for tracing the range of the furunculosis pathogen, *Aeromonas salmonicida* (Yoshimizu *et al.* 1992). A similar method was used in the case of roach *Rutilus rutilus* infected with the digenean, *Rhipidocotyle fennica* (Aaltonen *et al.* 1997).

Establishing the presence of antibodies does not establish that they confer immunity. Their effect on the disease can only be observed by passively immunising naïve fish with serum from fish exposed to a pathogen. Any non-specific immune

factors are likely to be present in the immunised fish in greater quantities than in the relatively small amount of serum injected. Consequently, if the immunised fish show enhanced resistance to the pathogen, specific antibodies are probably responsible for the resistance (Amend & Smith 1974, Harrell *et al.* 1975).

The principal aim of the present study was to establish whether wild striped snakeheads exhibit acquired immunity to EUS, and to examine the nature of the response if it was present.

Striped snakeheads were chosen as the study species, as more is known about their antibody response to *A. invadans* than the response of any other species (Thompson *et al.* 1997, Chapter 5, Chapter 6). Also, striped snakeheads are widespread and common throughout the Asia-Pacific region, and so are readily available.

In addition to information gleaned about the antibody response of striped snakeheads, the present study assessed three recently developed techniques. The first involved inducing EUS by immersion challenge with zoospores (Callinan 1997, Fairweather 1999). The second was the application of MAb 3gJC9 raised to *A. invadans* germlings to establish the presence of invasive *A. invadans* hyphae (Chapter 3, Chapter 4). The third was the use of the commercially available Rapi-diff stain (Section 2.2.5) as an alternative to H&E (Section 2.2.5) for counter-staining tissue sections after Grocott's methenamine silver stain (GMS) (Section 2.2.3).

The immersion challenge was necessary to replicate the natural mode of infection of *A. invadans*. Intramuscular injection of zoospores gives a reliable challenge (Wada *et al.* 1996, Thompson *et al.* 1997, 1999, Chapter 6), but natural *A. invadans* infections are unlikely to begin with high concentrations of zoospores already in the muscle. Injection bypasses the mucus, which contains many factors of the immune system (Shephard 1994), and is involved in the resistance of fish to saprolegniasis

(Wood *et al.* 1988). The skin itself functions as a purely physical barrier that excludes many oomycetes (Srivastava 1980), although there is evidence that it must be damaged before EUS can infect (Section 1.1.4).

Heavy rainfall and the consequent low pH of water have frequently been implicated in EUS outbreaks (Section 1.1.4), so previous immersion challenges have replicated the resulting dermatitis artificially (Callinan 1997, Fairweather 1999). Fairweather (1999) found that skin damage also admits *A. invadans*, so this was also incorporated into the challenge. Although such a combination of stress factors is more severe than is likely to occur simultaneously in a natural situation, it was necessary to maximise the potential for infection in order to have a sufficiently powerful study to establish whether the injected antibodies reduced infection. It was also intended that scraping the same area of skin on every fish would provide a clear point of entry for any infection, and so allow standardisation of sampling for histology.

Two methods of detecting *A. invadans* in fish tissue were carried out to compare IHC using MAb 3gJC9 with the standard GMS protocol (Section 2.2.3), and to ensure that the most powerful method for diagnosing EUS was used.

Rapi-diff has shown potential as a counter-stain as it has been used to stain *in vitro* preparations of macrophages and *A. invadans* (Thompson *et al.* 1999). The results suggested that it may show more detail in tissue sections than the commonly used light green counter stain (Chinabut & Roberts 1999) which does not allow clear recognition of the granulomata which are characteristic of EUS (Roberts *et al.* 1994). Although it is unlikely to show the same detail as H&E (Wada *et al.* 1994), it is much faster so it may have potential for use in routine diagnostics where large numbers of slides must be processed and fine detail is not required.

The present study intended to elaborate upon earlier work on the antibody response of fish to *A. invadans* (Chapter 5, Chapter 6) by establishing whether anti-*A. invadans* antibodies produced by fish vary in terms of total amount produced or their anti-*A. invadans* activity. Ultimately, the study was intended to evaluate the potential for the more practical applications of immunoprevalence monitoring, and protecting farmed fish by vaccination.

7.2 **Methods**

7.2.1 Immunoprevalence during EUS season

7.2.1.1 Collection of Fish

Wild striped snakeheads were sampled from a range of collection sites in the Philippines during the cold season in which EUS outbreaks occur. Sites were selected in order to examine as wide a range of epidemiological situations as possible (Table 7.1).

Most sites were on the island of Mindanao, as EUS first occurred there only two years prior to the study (Bureau of Fisheries and Aquatic Resources [BFAR] unpublished data). As endemic and non-endemic populations are found within a relatively small area (Fig. 7.1), it was possible to compare populations that were as similar as possible in other respects. Blood was sampled from the caudal vein within four hours of capture, and serum collected (Section 5.2.4).

7.2.1.2 Assessment of anti-*A. invadans* relative antibody concentration (RAC)

RAC was quantified by ELISA on soluble antigens derived from 1d germlings of isolate B99C (Section 5.2.8). Dilutions of fish serum ranged from 1:32 to 1:32768 in antibody buffer.

Table 7.1. Sites of collection of fish for seroprevalence study. Information of EUS status in Philippines from BFAR records.

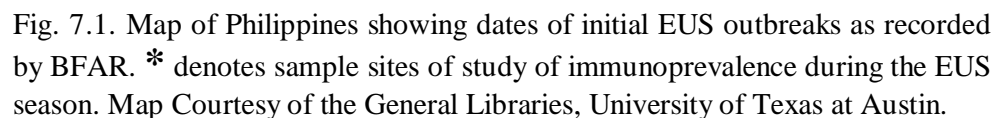
Group	Capture date	Location	EUS status of site	Number of samples
A	05/10/99	Upper reaches of River Polangi, Bukidnon Province, Region X, Mindanao, Philippines.	EUS never recorded	15
B	05/10/99	Tilapia grow-out pond in Barongot, Dancagon, Bukidnon Province, Region X, Mindanao, Philippines.	EUS never recorded	8
C	08/01/00	Lake Buluan, Sultan Kudarat Province, Region XI, Mindanao, Philippines	Sampling during first recorded EUS outbreak in Lake Buluan.	11
D	21/12/99 and 07/01/00	Bunawan, Agusan marshes, Agusan del Sur, Region XI, Mindanao, Philippines.	EUS first recorded in January 1999.	13
E	07/01/00	Carmen, Davao del Sur Province, Region XI, Mindanao, Philippines.	Sampling during first recorded EUS outbreak in Carmen.	7
F	02/02/00	Rice field in Muñoz, Isabela Province, Region II, Luzon, Philippines.	EUS recorded since 1989. Samples collected during an outbreak.	11

7.2.1.3 Specificity of antibody response

Specificity of the antibody response was assessed by comparing the RAC (Section 5.2.8) against *A. invadans* isolate B99C to the RACs against two non-invasive *Aphanomyces* spp., ASEAN1 and WSA, and a non-invasive *Saprolegnia* sp., TF20S (Table 2.1) as antigens. Serum dilutions ranged from 1:16 to 1:16384.

7.2.1.4 Fungicidal Assay

The ability of serum to inhibit the germination of the cysts of *A. invadans* isolate B99C (Table 2.1) was assessed, as previous studies indicated that antibody levels are more related to inhibition of germination than growth (Section 5.2.5). The technique was further adapted in that wells were counted in quintuplicate rather than triplicate to provide more data for each individual.



7.2.1.5 Statistical analysis

Total anti-*A. invadans* RACs were compared by 1-way ANOVA. Specificity of the antibody response was compared by nested 2-way ANCOVA with interaction. Fungicidal assays were compared by performing nested ANOVAs on indices of inhibition (Section 5.2.9).

All significant ANOVAs were followed by Tukey multiple comparisons. Test values of $p < 0.05$ were considered significant. Test statistics are presented where results refer to only one test. Where results refer to more than one test, the critical value given refers to the highest present in any case.

7.2.2 Comparison of antibody activity between seasons

7.2.2.1 Collection of fish

Striped snakeheads were collected from two pond farms in Suphanburi Province, Central Thailand. The first collection at each site was carried out in May 1999, outside the EUS season, and the second in February 2000, during the EUS season. Sample sizes ranged from 20-24 fish per sample group.

Central Thailand has been EUS-endemic since 1981 (Tonguthai 1985), and some outbreaks were recorded in early 1999 (Lilley *et al.* in press), though it is not known whether EUS had occurred on the sampled farms. Both farms were stocked with wild fingerlings caught locally. Both farms are routinely stocked in September – October every year, and harvested completely by June.

7.2.2.2 Examination of sera

The total anti-*A. invadans* RAC, antibody specificity and inhibitory activity of the sera were assessed as previously described (Sections 7.2.1.2, 7.2.1.3, 7.2.1.4).

7.2.2.3 Statistical analysis

Total anti-*A. invadans* RACs and inhibitory activity were compared by 2-way ANOVA with interaction. All other analysis was performed as previously described (Section 7.2.1.5).

7.2.3 Passive Immunisation

7.2.3.1 Experimental Fish

Wild striped snakeheads were caught from the upper reaches of the River Polangi, near the group A sample site (Section 7.2.1.1). BFAR has monitored the area continuously, and no EUS has ever been reported from the Polangi (Fig 7.1, Table 7.1).

Eight fish were stocked per 40l tank in duplicate tanks per treatment. They were acclimatised for 10d at a temperature of 24°C ±1°C. Fish were fed daily to satiation on live Nile tilapia, guppy *Poecilia reticulata* or goldfish *Carassius auratus* fingerlings.

There was considerable variation in size of the fish available (\bar{x} = 69.7g, sd = 25.8), so they were allocated to tanks in such a way as to give size distributions between tanks that were as similar as possible.

7.2.3.2 Serum treatments

Three serum treatments were collected from different groups of snakeheads (Table 7.2).

Serum 1 was collected from the same population as the experimental fish and used as a control.

Serum 2 was collected from the Suphanburi, Thailand population that was used in previous chapters, and was found to express antibodies against *A. invadans* (Sections 5.3.10, 6.3.2.5). Serum 2 was collected from fish after injection challenge with *A. invadans* isolate B99C.

Serum 3 was from a Philippines population from which EUS was not reported until November 1999, a month prior to the study, and considered to be EUS free before that date.

The RAC, cross-reactivity and inhibitory activity of all sera were examined as previously described (Sections 7.2.1.2, 7.2.1.3, 7.2.1.4).

Table 7.2. Source of serum treatments used for passive immunisation.

Treatment	Source
1	Wild fish, River Polangi, Bukidnon Province, Mindanao, Philippines.
2	Fish injected with <i>A. invadans</i> isolate B99C, collected from Suphanburi Province, Thailand.
3	Wild fish recovered from natural EUS outbreak, Iloilo Province, Panay, Philippines

7.2.3.3 Passive immunisation and challenge

Each fish was injected intraperitoneally with 50µl of serum, and immediately challenged with *A. invadans* by immersion by a protocol adapted from Callinan (1997) and Fairweather (1999).

The fish were subjected to acid stress by placing in 5g l⁻¹ NaH₂PO₄ (BDH) for 30min, and an area of skin was exposed by removal of scales immediately to the right of the anterior dorsal fin.

The fish were then replaced in the 40l tanks along with 14 mats of *A. invadans* isolate B99C that had been prepared and washed (Section 2.1.3). The mats were left to sporulate in the tanks.

7.2.3.4 Sampling protocol

An *ad hoc* sampling protocol was used. All moribund fish were sampled and the day noted. After 30d, all remaining fish were sampled.

When sampled, fish were weighed and a section of the muscle immediately below the area of exposed skin was excised and fixed for histology (Section 2.2.2).

7.2.3.5 Histology

Histological analysis was carried out both by indirect IFAT with MAb 3gJC9 (Chapter 4) and with GMS (Section 2.2.3) in order to compare the results from the different techniques. Sections stained with GMS were counter stained with Rapi-diff (Section 2.2.5) in order to assess it as an alternative to H&E for basic histopathology.

Both sets of slides were assessed for the presence or absence of invasive hyphae, and those stained with Rapi-diff were used to assess the development of the inflammatory response (Section 6.2.2.5).

7.2.3.6 Statistical analysis

The total anti-*A. invadans* RACs of the sera used for passive immunisation were compared by 1-way ANCOVA with interaction. Specificity was compared by 2-way ANOVA. The inhibitory activity of the sera was compared by 1-way ANOVA.

The weights of fish were compared between the tanks by 1-way ANOVA to ensure that they were evenly distributed.

The numbers of infected and uninfected fish were compared between treatments by Pearson λ^2 test for independence of association.

Survival was compared between treatments by Cox regression, taking into account the weights of fish.

Areas of total inflammatory response, acute inflammation, granuloma formation, and the ratio between acute inflammation and granuloma formation were compared by ANCOVA using weight and time as covariates.

Test values of $p < 0.05$ were considered significant. All ANOVAs and ANCOVAs were followed by Tukey multiple comparisons if significant. Test statistics are presented where results refer to only one test. Where results refer to more than one test, the critical value given refers to the highest present in any case.

7.3 Results

7.3.1 Immunoprevalence during EUS season

7.3.1.1 Anti-*A. invadans* RAC

Anti-*A. invadans* RACs of fish collected from different sites during the EUS season were compared and differences were found ($F = 11.65$, $df = 5$, $p < 0.0005$).

The group C sera, collected during the first recorded outbreak in Lake Buluan,

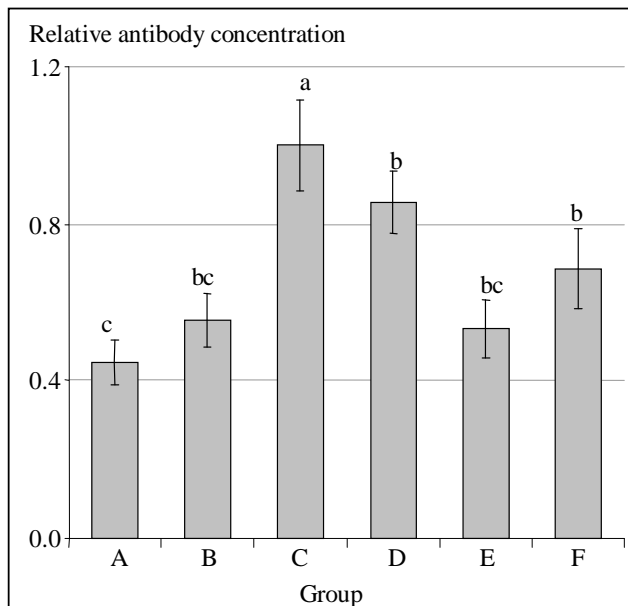


Fig. 7.2. Anti-*A. invadans* Relative antibody concentrations of striped snakehead from sample sites in the Philippines during winter of 1999-2000. Letters indicate significance groups. Bars indicate 95% confidence intervals. Groups defined in Table 7.1.

showed higher RACs than those of any other group ($p < 0.01$).

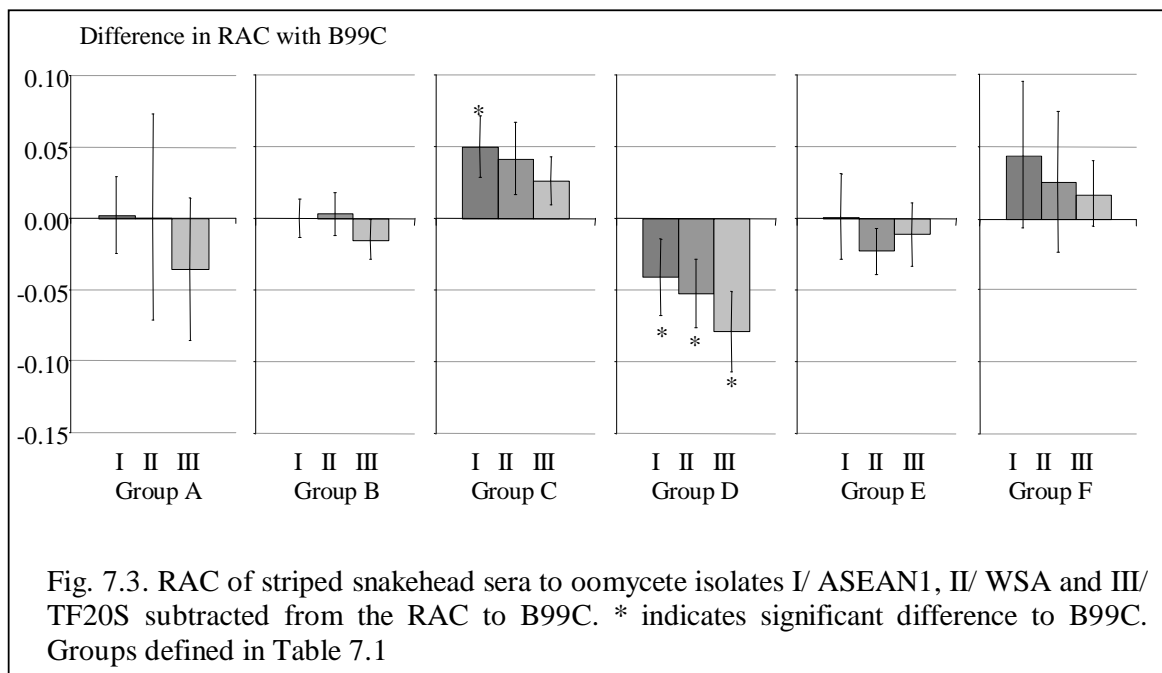
The RACs of groups A and B, the EUS-naïve fish, were among the lowest, although there were groups with higher RACs that were not significantly different to them (Fig. 7.2).

7.3.1.2 Specificity of the antibody response

The RACs against *A. invadans* isolate B99C were compared to

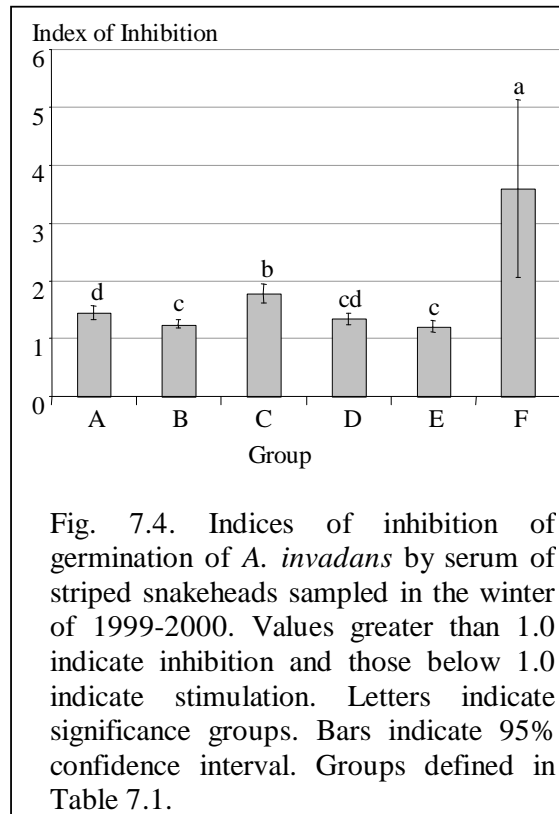
those obtained with two other *Aphanomyces* spp, ASEAN1 and WSA, and a *Saprolegnia* sp, TF20S.

Although the RAC to *Saprolegnia* isolate TF20S was lower than the RAC to all of the *Aphanomyces* spp. across all sample groups ($p < 0.05$), the only single group in which it was lower was group D ($q = -7.19$, $p < 0.0005$), which was undergoing its second EUS season. The RAC to the other two *Aphanomyces* spp. was also lower in group D ($p < 0.05$). The only other significant difference was in group C, which was undergoing its first EUS outbreak, where the RAC to *A. laevis* isolate ASEAN1 was higher than *A. invadans* isolate B99C ($q = 4.16$, $p < 0.05$) (Fig. 7.3).



7.3.1.3 Inhibition of germination

Inhibition of the germination of *A. invadans* was compared between the sample groups and differences were found ($F = 5.42$, $df = 5$, $p < 0.005$). Group F, the sample collected from Luzon, was the most inhibitory by a considerable magnitude ($p < 0.00005$) (Fig. 7.4).



7.3.2 Comparison of antibody activity between seasons

7.3.2.1 Anti-*A. invadans* RAC

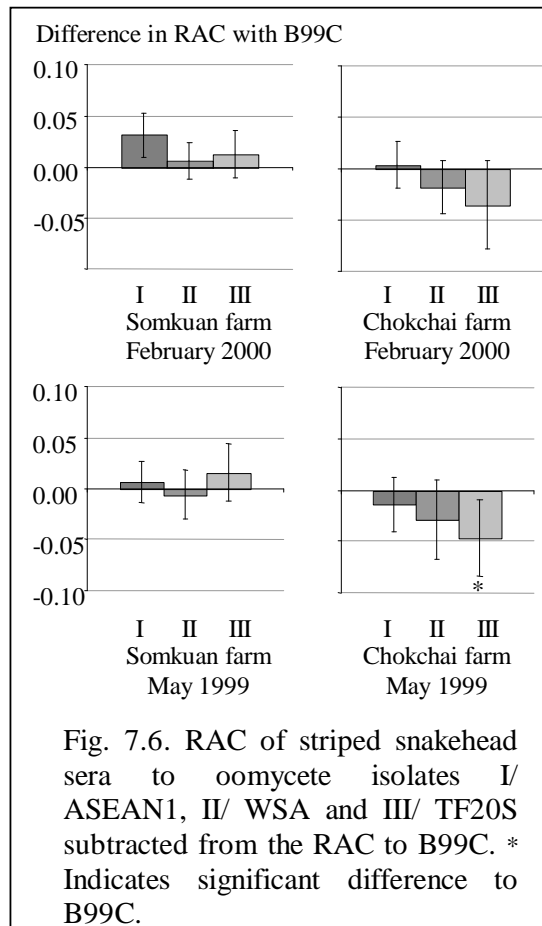
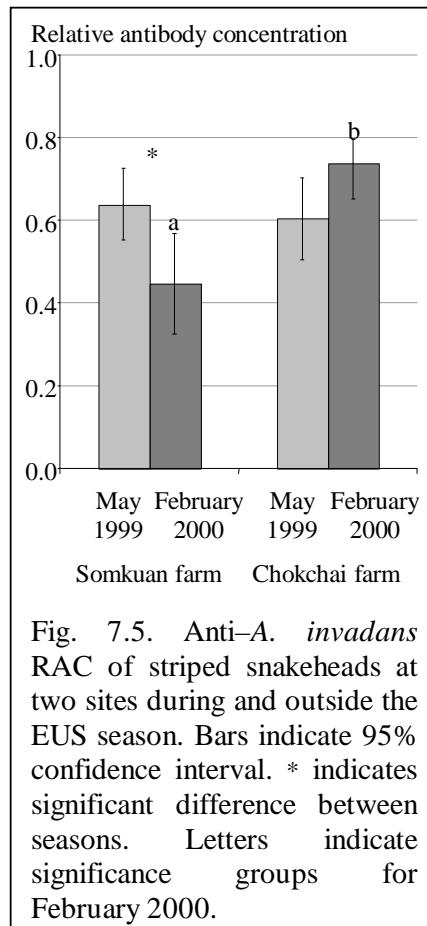
Anti-*A. invadans* RACs were compared between samples collected from two different sites in an EUS-endemic region of Thailand, during the EUS season and outside it.

Differences were found ($F = 12.51$, $df = 1$, $p < 0.005$) between Somkuan and Chokchai farms in February ($q = -4.29$, $p < 0.0005$). Fish at Somkuan farm had higher RACs in February than May ($q = 3.22$, $p < 0.01$) (Fig. 7.5).

7.3.2.2 Specificity of the antibody response

The RAC of sera sampled from fish in Thailand, collected at different sites and different seasons, to *A. invadans* isolate B99C was compared to the RAC of three other oomycetes. No differences were found between sample groups, ($F = 2.28$, $df = 3$, $p > 0.05$), though there were differences between the RACs to the different antigens across all samples ($F = 7.37$, $df = 3$, $p < 0.0005$).

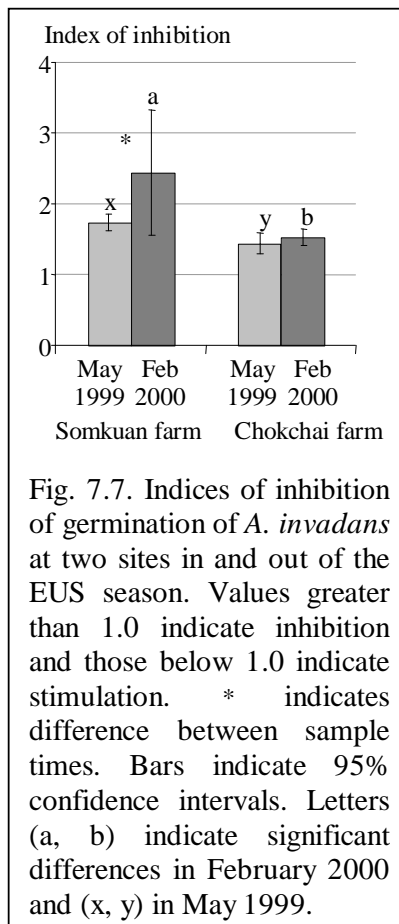
Saprolegnia sp. isolate TF20S elicited a lower RAC than *A. invadans* isolate B99C or *A. laevis* isolate ASEAN1 across all the samples ($p < 0.005$). However, there was only one difference within any one group, which was in the case of Chokchai farm in February, where the RAC to *A. invadans* isolate B99C exceeded the RAC to *Saprolegnia* sp. isolate TF20S ($q = -4.68$, $p < 0.0006$) (Fig. 7.6).



7.3.2.3 Inhibition of Germination

The ability of sera from Thai fish, collected at different sites and during different seasons, to inhibit the germination of *A. invadans* was compared.

Sera from Somkuan farm were more inhibitory than those from Chokchai farm ($F = 62.92$, $df = 1$, $p < 0.0005$), and sera collected in February were more inhibitory than those collected in May ($F = 14.94$, $df = 1$, $p < 0.0005$) (Fig. 7.7).



7.3.3 Passive Immunisation

7.3.3.1 Weights

There was no difference in the weight distributions of the different tanks ($F = 0.25$, $df = 5$, $p > 0.05$).

7.3.3.2 Analysis of sera

The RACs, cross-reactivity and ability to inhibit the germination of *A. invadans* of the sera used for passive immunisation were examined.

The anti-*A. invadans* RACs of sera 1 and 2 were similar ($q = 0.29$, $p > 0.05$), but that of serum 3 was higher than either ($p < 0.05$) (Fig. 7.8a).

There was no difference in the RAC elicited by the different antigens either across all three sera

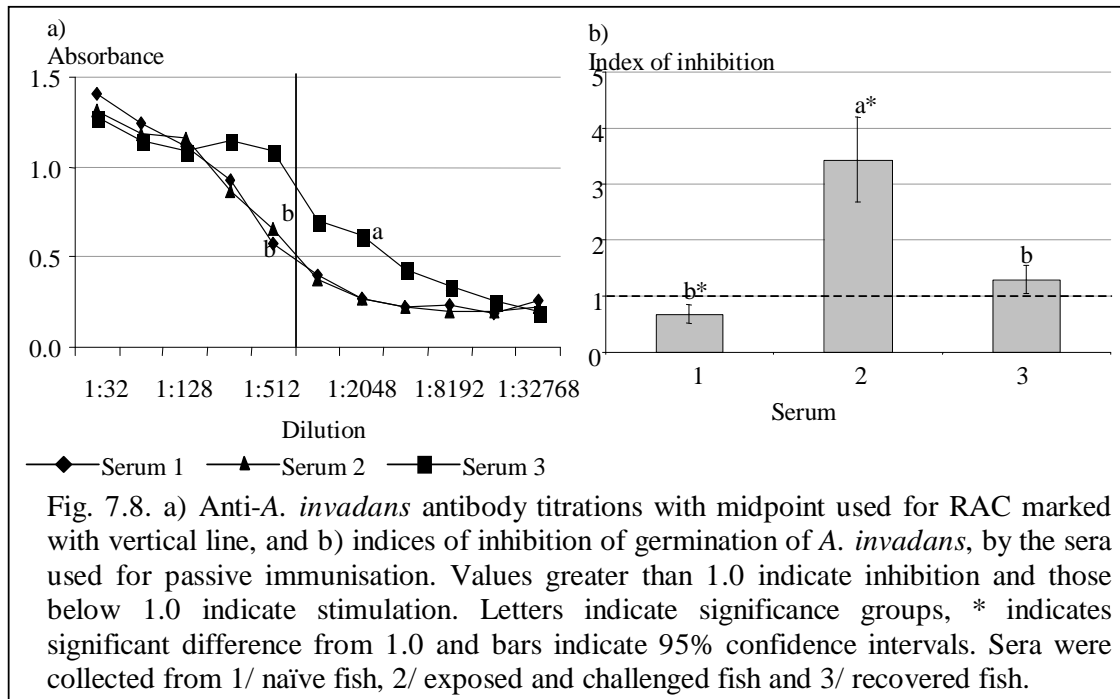
($F = 2.67$, $df = 3$, $p > 0.05$) or within any one serum ($F = 0.81$, $df = 6$, $p > 0.56$).

Only serum 2 was inhibitory ($q = -7.38$, $p < 0.0005$), while serum 3 had no significant effect ($q = -1.22$, $p > 0.05$) and serum 1 increased germination ($q = 3.07$, $p < 0.05$) (Fig. 7.8b).

7.3.3.3 Incidence and Mortality

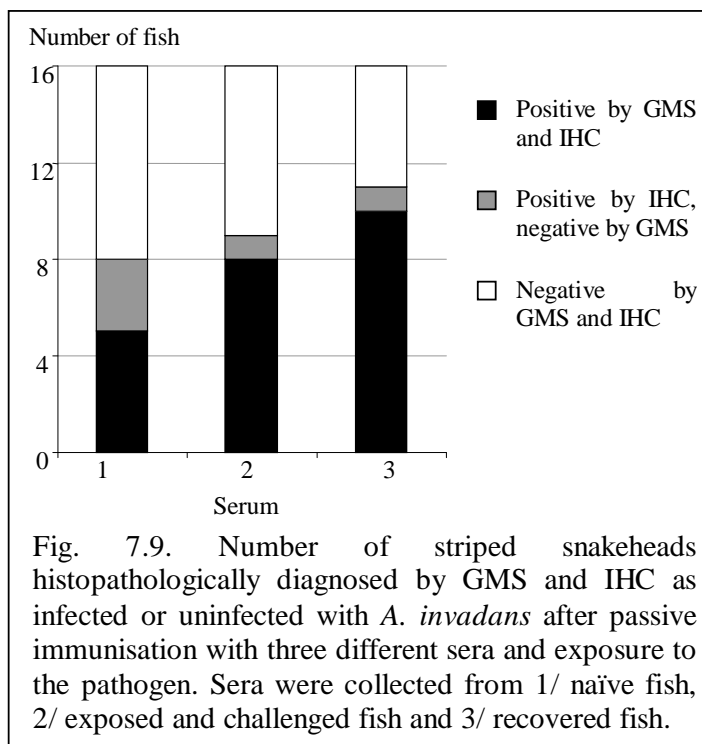
Times taken after challenge for fish to become moribund were noted, and the fish were subjected to histopathological examination with GMS and IHC with MAb 3gJC9 to establish the presence or absence of *A. invadans* hyphae.

IHC was found to be more sensitive than GMS in detecting invasive hyphae, but there was no difference in the incidence of infection between the three groups whether



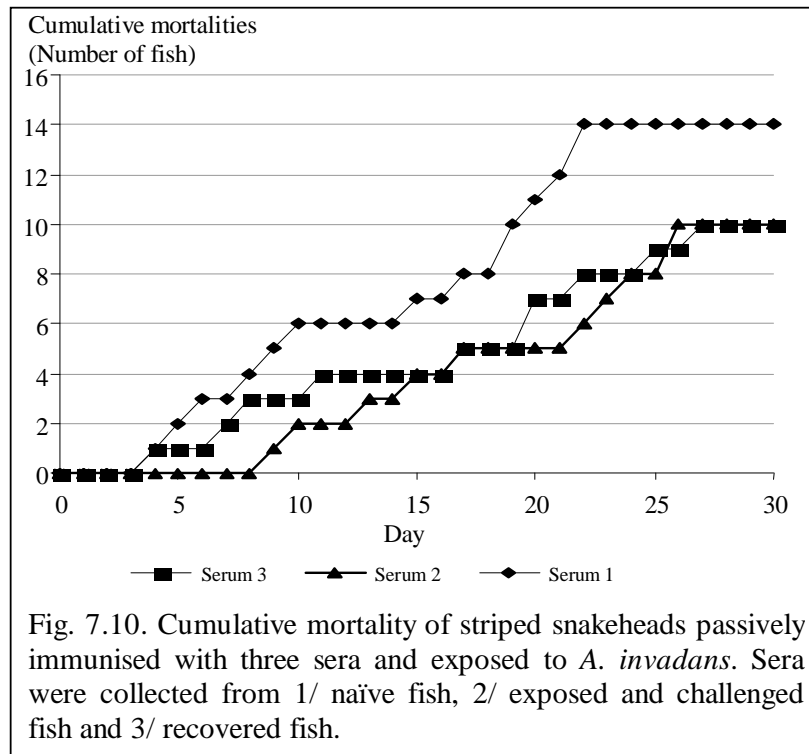
assessment was made by IHC ($\lambda^2 = 3.17$, $df = 2$, $p > 0.05$) or GMS ($\lambda^2 = 2.16$, $df = 2$, $p > 0.05$) (Table 4.2, Fig. 7.9).

The mortality of fish passively immunised with serum from fish injected with *A.*



invadans (Serum 2) was less than that of fish immunised with serum from naïve fish (Serum 1) (Wald statistic = 5.22, $df = 1$, $p < 0.05$). Mortality of fish injected with the serum of recovered fish (Serum 3) was also lower than that of fish injected with naïve serum (Serum 1), though statistical significance was

equivocal (Wald = 3.040, df = 1, $p = 0.08$) (Fig. 7.10).



7.3.3.4 Development of the Inflammatory Response

The extent and development of the inflammatory response were compared between fish immunised with different sera.

Rapi-diff was found to be an adequate counter-stain for the level of histopathological detail required (Fig. 7.11), though it did not elucidate as much detail as H&E (Fig. 6.1).

The areas of chronic inflammation, granuloma formation, the ratio of the two and the total area of the inflammatory response of infected fish were compared between groups, and covariance with weight or the day of sampling was assessed.

No difference between groups was found in any case ($p > 0.05$). There was positive covariance of the area of granuloma formation and sample day ($F = 6.17$, $df = 1$, $p < 0.05$), and between the ratio of the areas of acute inflammation: granuloma formation and sample day ($F = 6.38$, $df = 1$, $p < 0.05$).

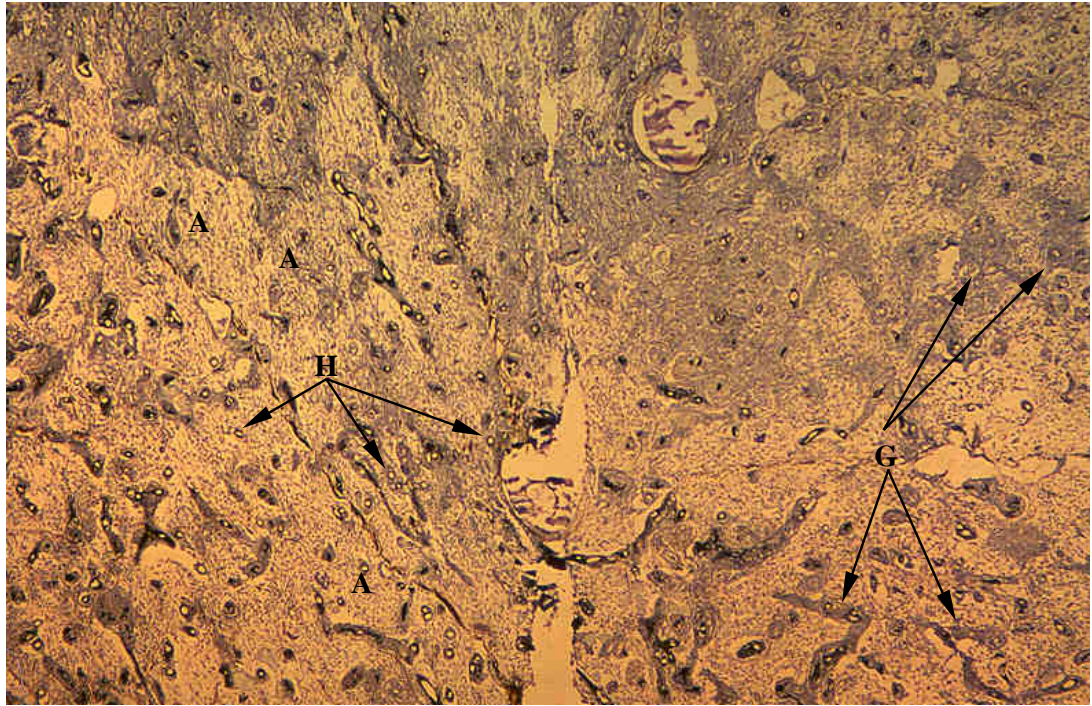


Fig. 7.11. Muscle section of moribund striped snakehead 26d after immersion challenge with *Aphanomyces invadans* illustrating hyphae (H), granulomata (G) and acute inflammation (A). Stained with GMS and rapi-diff, viewed at 40x magnification.

7.4 Discussion

The antibody response to *A. invadans* in striped snakeheads was compared between populations with regard to the seasonality and the geographical range of EUS. In addition, the techniques of immersion challenge with *A. invadans*, IHC for histopathological diagnosis and Rapi-diff staining for elucidation of histopathological detail were evaluated.

The comparison of acquired immunity between populations would have been more powerful if samples had been taken sequentially over the course of an entire year, but such a schedule was not possible due to logistical constraints.

The specificity of the antibodies present in the sera revealed very little about the nature of the response. Even the most inhibitory groups among the samples taken in the Philippines (Groups C and F) and Thailand (Somkuan farm, February 2000) showed

little difference in their response to *A. invadans* when compared to their response to other *Aphanomyces* spp. Considerable antibody cross-reactivity between *Aphanomyces* spp. has been observed in studies of rabbit PABs (Petersen *et al.* 1996, Lilley *et al.* 1997c), so the lack of specificity of snakehead antigens is unremarkable.

A non-specific antibody response to certain hyphal pathogens has been observed in mammals (Ahmed 1982, Lehmann 1985), as has the presence of anti-*A. invadans* antibodies in EUS-naïve rainbow trout (Thompson *et al.* 1999). Similarly, non-specific antibodies are evidently present in EUS-naïve striped snakehead populations, and antibodies that inhibit *A. invadans* also recognised other oomycetes such as the *Saprolegnia* sp. isolate TF20S.

The ubiquity of oomycetes (Srivastava 1980) and the opportunistic nature of many of them (Section 1.4.1) suggests that fish may have developed an antibody response to them, which may cross-react with taxonomically similar pathogens. A similar observation was recorded during a study of the immunoprevalence of the response of European badgers *Meles meles* to the bovine tuberculosis pathogen, *Mycobacterium bovis*. An antibody response to *M. bovis* was found in naïve badgers due to their exposure to non-pathogenic *Mycobacterium* spp. in the environment (Stainsby *et al.* 1989). Specificity has not been assessed in the antibody responses of fish, but a study of anti-*Aeromonas salmonicida* antibodies in salmonids found that many more sera contained such antibodies than demonstrated agglutinating activity (Yoshimizu *et al.* 1992). The lack of activity suggests that the antibodies may have been a response to other *Aeromonas* spp, and do not indicate exposure to *A. salmonicida*.

EUS-naïve populations (Groups A and B) exhibited the lowest specific anti-*A. invadans* RAC, along with a population that was undergoing its first EUS outbreak (Group E). In the latter case, it was not known how long the outbreak had been

occurring for, but the fish were sampled about three months into the normal season so they may not have had time to develop a complete antibody response. By contrast, the other population undergoing its first outbreak (Group C) appeared to have mounted such a response as it had the highest anti-*A. invadans* antibody level. Unfortunately, precise information regarding how long the two outbreaks had been in progress at the time of sampling is not available.

With the exception of Group E which was undergoing its first outbreak, all the exposed populations had higher specific RACs than either naïve group, although they were only significantly different from both in the case with the highest level (Group C).

The only significant seasonal difference in specific RAC found in the seasonal study was in the case of Somkuan farm, where RACs were higher in the warmer season. Such a result is to be expected in the light of the temperature-mediated differences in antibody production (Chapter 4), although it was not reflected on Chokchai farm.

In spite of the relatively low specific RAC of Group F, from Luzon, in comparison to that of other populations exposed to EUS, its ability to inhibit germination was considerably greater than that of any of the Mindanao populations. Similarly, the highest inhibitory activity among the Thai sera was found in those collected from Somkuan farm in February, which had the lowest RAC.

The sera used for the passive immunisation trial followed the same trend. The serum from Panay fish that had recovered from their first exposure to *A. invadans* had the highest specific RAC, although it was considerably less inhibitory or protective than the serum from recently challenged Thai fish.

The fact that sera from Luzon and Thailand were more inhibitory than is indicated by the specific RACs, implies that these fish were producing a more efficient response than the recently exposed Mindanao fish. Such an improvement in the nature

of the response may occur within individuals over long periods of exposure to *A. invadans*.

Several studies on salmonids have observed changes in the nature of the antibody response during long term exposure to an antigen. Arkoosh and Kaattari (1991) observed changes in specificity of rainbow trout antibodies, which implied that different antigens may be recognised. Improvements have been reported in the affinity of Atlantic salmon antibodies to their antigen in a similar manner to the affinity maturation that is characteristic of the secondary immune response of mammals (Killie *et al.* 1991), although Arkoosh and Kaattari (1991) specifically reported the absence of improved affinity. Killie *et al.* (1991) also found changes in the binding of anti-salmon Ig MAbs over the course of their trial, and suggested that a process analogous to the switching of the predominant isotype, observed in mammal antibody responses, may have been taking place. However, the existence of different antibody isotypes has never been conclusively shown in fish, although Elcombe *et al.* (1985) found that rainbow trout Ig occurred both as a tetramer and a monomer.

Any or all of the processes of antigen changing, affinity maturation or isotype switching may be responsible for the improved immunity in populations exposed for longer periods of time observed in the present study. However, Arkoosh and Kaattari (1991) concluded that enhanced immunity after long-term exposure was due largely to the development of a larger clone of antibody producing cells, and the consequent production of more antibodies. Such a mechanism cannot be responsible for the enhanced immunity observed in snakeheads, as the RACs bore no relationship to the fungicidal or protective capabilities of the sera.

An attempt was made to elucidate the differences in the antibody response between groups by Western blot analysis (Section 3.2.4.2), in order to find out whether

antigen changing occurred. However, the results of preliminary experiments were unclear and inconsistent, so this approach was not pursued.

As the differences in immunity were noted between populations that were only sampled once, it is possible that they were not due to changes occurring within individuals but to selection within the populations. The mortalities that are often associated with early outbreaks of EUS are of such a scale (Tonguthai 1985, ACIAR 1998) that any individuals unable to mount an effective immune response may be eliminated from the population. Major fishkills are often observed during the first few EUS seasons (Tonguthai 1985, Chinabut 1998), so individuals with a less efficient immune response may survive the first season to die in subsequent outbreaks. If so, populations would not show a particularly effective response after only one outbreak, as was observed in the present study (Group D). The possibility of selection for resistance to specific pathogens within fish populations has been discussed before, such as in the variation in the resistance of rainbowfish *Melanotaenia* spp. from different populations in their resistance to ichthyophthiriasis (Gleeson *et al.* 2000), but not specifically linked to the acquired immune system.

It is possible that selection may take place even if there is an improvement in the efficiency of the response within individuals, as not every individual may be able to produce the antibodies necessary to provide protection to repeated outbreaks. Alternatively, individuals that are able to produce the necessary antibodies may not survive for long enough for their antibody response to mature if their innate immune mechanisms lack the ability to control early infections (Chapter 5, Chapter 6). Selection caused by EUS would be a cause for some concern in terms of its impact on diversity within populations, as the high mortalities caused by the initial outbreaks of EUS may cause a genetic bottleneck within the population.

It is unlikely that selection is responsible for the fact that EUS-exposed populations showed an increase in inhibitory activity during the EUS season with no increase or an actual decrease in the RAC. It is possible that more antibodies are produced to the antigens that confer protection when the population is exposed to EUS, but the lack of change in the specificity of the response makes this unlikely. Alternatively, the increase in inhibitory activity may be due to an increase in a factor other than antibodies, such as complement, which earlier studies indicated is a major factor in the inhibition of germination by low temperature snakehead serum (Chapter 5). The improvement in immunity in exposed populations makes it unlikely that the increase in inhibition was conferred by complement alone, but complement may have been acting synergistically with antibodies through the antibody mediated classic complement pathway.

While a relationship between antibody production and inhibitory activity is implied by the data collected from the Philippines and by previous studies (Chapter 5), it is necessary to consider the relationship between inhibition and protection before firm conclusions may be drawn. Unfortunately, the assessment of the protective activity of the antibodies was hindered by a number of logistical and methodological difficulties.

Some such difficulties were encountered in obtaining fish, and it was necessary to use fish with a much greater size distribution than was desirable. This was a particular problem as many of the fish were in breeding condition at the time the experiment was carried out, so larger fish defended territories within the tanks. Studies on rainbow trout (Peters *et al.* 1988, 1991) and hybrid tilapia *Oreochromis mossambicus* x *Oreochromis urolepis* (Faisal *et al.* 1989) have found that social stress reduces immunocompetance among subordinate fish. Although no such studies have been carried out among snakeheads, a similar stress response is the most likely reason

for fish size being the most significant determinant of mortality in the course of the experiment. Due to the aggressive behaviour of the fish, it was not possible to stock fish at the densities that were anticipated from previous experience (Sections 5.2.1, 6.2.1.1, 6.2.2.1), which led to sample sizes being considerably smaller than was desirable.

The immersion challenge protocol was successful in that it gave infection rates comparable to those observed in injection challenge (Chapter 6), but it created difficulties in sampling procedures. Infection other than at the scraped site was only observed in one fish where there was no lesion at the scraped site, but it may have been far more prevalent than was detected. This appears particularly likely as mortality rates reflected those that would be expected according to the injected sera, although apparent infection rates bore no resemblance to those that would be expected. Future assessments of immersion challenges should be assessed by taking several samples along the length of the dorsal muscle.

In spite of the limitations of the challenge procedure and available fish, the serum derived from the exposed and challenged fish (Serum 2) was found to be more inhibitory than EUS-naïve serum (Serum 1), and to improve survival when used for passive immunisation. The serum from recovered fish from Panay (Serum 3) also appeared to have some defensive ability, as the mortality by the end of the trial was the same as that in the case of fish immunised with the serum from challenged Thai fish (Serum 2). The fact that the mortalities among fish immunised with serum 3 occurred earlier in the trial than in the case of fish immunised with the more inhibitory serum (Serum 2) suggests that it afforded little protection in cases where *A. invadans* infection took hold.

Assessment by IHC recorded the presence of *A. invadans* in several cases where it was not clearly visible when stained by GMS. These results suggest that it is probably

the more sensitive of the two stains, and may allow the recognition of EUS at a much earlier stage (Chapter 4).

Although no differences were observed in the formation of the granulomatous response, the study did show that Rapi-diff is a viable alternative to H&E as a counter-stain to GMS. Rapi-diff had the advantage of allowing far more rapid processing, and although it does not give the same level of cellular detail as H&E, it was adequate for basic histopathology. It may be recommended for basic diagnostic use where many samples must be processed in order to determine the presence of mycotic granulomata, and so establish the case definition of EUS (Roberts *et al.* 1994).

While the fluctuating relationship between antibody production and the length of time for which the populations have been exposed to EUS is of some interest, it makes it unlikely that immunoprevalence monitoring has any potential for tracking the spread of *A. invadans*. However, the high RACs characteristic of the earlier outbreaks within the region may enable fisheries inspectors to attribute an outbreak in a hitherto non-endemic region to EUS without the need to identify *A. invadans* directly.

The confirmation that the anti-*A. invadans* antibodies expressed by snakeheads are protective indicates that vaccination may be technically possible. However, the logistical difficulties and costs of vaccination may be prohibitive in most Asian freshwater systems.

7.5 Summary

- Antibodies raised to *Aphanomyces* spp. cross-react strongly within the genus, and to a substantial degree with other oomycetes.
- Strong inhibition of *A. invadans* germination by serum corresponds to protective activity.

- Total RAC and specificity of RAC do not correspond to inhibitory or protective activity.
- Populations recently exposed to EUS develop a high anti-*A. invadans* RAC.
- Populations that have been previously exposed to EUS have a more protective antibody response, although their RACs are not necessarily high.
- EUS outbreaks can be successfully replicated using the immersion challenge protocol, but a standardised histopathological sampling protocol should be developed.
- IHC with MAb 3gJC9 is a more powerful diagnostic technique than GMS.
- Rapi-diff is a viable alternative to H&E for histopathological examination, although it does not show as much detail.
- Immunoprevalence monitoring is unlikely to be appropriate for long term monitoring of the range of EUS, but may provide a rapid method of confirming the involvement of *A. invadans* in outbreaks in previously non-endemic areas.
- Protection against EUS may be conferred by vaccination, although it may not be appropriate due to financial and logistical constraints.

8 General Discussion

8.1 Infection and the Inflammatory Response

The pathology of EUS is often described with reference to the inflammation it causes, which follows a remarkably consistent sequence in taxonomically distinct hosts (Callinan *et al.* 1989, Viswanath *et al.* 1997, Lilley *et al.* 1998). The use of IHC in the present study elucidated elements of the process of infection by *A. invadans* that have not been previously recorded, and the studies performed on granulocytes *in vitro* and *in vivo* supplemented what was already known on functional aspects of the cellular immune response.

8.1.1 Initial Infection

Many previous reports on the pathology of EUS have described naturally infected fish which were sampled because they had visible lesions, indicating that pathology was already well developed (McKenzie & Hall 1976, Wada *et al.* 1994, Vishwanath *et al.* 1997). Others assessed pathology after injection with *A. invadans*, placing large numbers of zoospores directly into fish muscle tissue (Catap & Munday 1998a, Khan *et al.* 1998, Thompson *et al.* 1999). Evelyn (1996) considered the ‘ability to attach to a host . . . an important prerequisite for the successful establishment of an infection’, but the way by which a pathogen gains entry into the host cannot be studied by either of these two methods

The successful induction of EUS by immersion challenge (Callinan 1997, Fairweather 1999) raised the possibility of reproducing a natural outbreak under controlled conditions, and examining the very early stages of such an infection. Although no study has been carried out with this specific intention, IHC of fish that

became moribund after immersion challenge (Chapter 4, Chapter 7) allowed detection of *A. invadans* at a much earlier stage of infection than has previously been reported.

The muscle fibres of such fish were infiltrated with what appeared to be much narrower hyphae than have been previously observed. These structures were sometimes found in muscle tissue close to the more commonly reported larger hyphae in the dermis (Egusa & Masuda 1971, McKenzie & Hall 1976). The fact that there were stages of the mycelium that were not recognised by GMS is not unprecedented, as Viswanath *et al.* (1997) found fish from EUS-outbreaks with ‘pinhead sized’ EUS-type lesions, and characteristic pathology, in which no hyphae were observed by GMS. These observations may be explained if GMS does not stain the earliest infective stage, as the present study indicates. The presence of hyphae in the dermis would not indicate a diagnosis of EUS to a pathologist viewing sections stained with GMS, as they do not conform to the definition of ‘invasive’ that characterises *A. invadans* (Roberts *et al.* 1994).

The lack of cross-reactivity of MAb 3gJC9 to other oomycetes found in fish enabled a distinction to be made between *A. invadans* and opportunists in the dermis which is not possible using GMS. The putative early stage of the hyphae was not associated with necrosis or inflammation except in cases where more advanced hyphae had also penetrated the musculature. There is probably a stage of infection between that recorded here and the stage reported by Viswanath *et al.* (1997) that was not seen in the present study as samples were not taken at regular intervals.

8.1.2 Early Infection and Inflammation

The widespread necrosis and acute inflammation that characterises the early stages of *A. invadans* infection have been widely reported (Callinan *et al.* 1989, Lilley & Roberts 1997, Viswanath *et al.* 1997), although the causal mechanisms are not known. ECPs

that cause similar effects have been identified in infections with other oomycete pathogens such as *A. astaci* (Söderhäll & Unestam 1975, Söderhäll *et al.* 1978) and *Saprolegnia* spp. (Peduzzi & Bizzozero 1977). The importance of ECPs in EUS may be inferred by the fact that necrosis is commonly observed some distance away from the hyphae (McKenzie & Hall 1976, Wada *et al.* 1994), and the findings of the present study indicates that such necrosis is caused by ECP released by the mycelium (Chapter 4).

A. invadans possesses haemolytic and haemagglutinating factors that are unlikely to be functional unless expressed extracellularly (Kurata *et al.* 2000a), though the mode of expression has never been established. Electron microscopy of interactions between rainbow trout macrophages and *A. invadans in vitro* showed secretions that appeared to be passing from *A. invadans* hyphae into the macrophages, although the exact nature of the interaction was unclear (Thompson *et al.* 1999). In spite of the considerable evidence for the involvement of ECPs in the pathogenicity of *A. invadans*, their existence was not conclusively proved until PABs raised to ECPs in broth culture medium were used to detect antigens in hyphae (Lilley *et al.* 1997c).

Four of the five MABs in the present study recognised antigens that were expressed extracellularly (Chapter 3). The same MABs bound to the surface of 1d germlings, implying that much of the antigen present on the surface of germlings is retained or in the process of being secreted.

IHC revealed that the antigen recognised by MAB 3gJC9 was found diffused throughout much of the necrotic tissue, and was not present where the muscle fibres were undamaged (Chapter 4). Although the function of the specific antigen recognised is not clear, this observation supports the suggestion that ECPs destroy tissue to pave the way for hyphal infiltration, in a similar way to the ECPs of *A. astaci* (Söderhäll *et*

al. 1978). The putative early stage of the mycelium associated with the first stage of infection (Section 8.1.1) was also found in the necrotic tissue, some way ahead of the developed hyphae and ECP infiltration

Many oomycete saprophytes and plant or animal parasites secrete enzymes to digest their hosts or substrates extracellularly, either to remove physical barriers to their spread or to break down nutrients for absorption (Unestam 1966, Peduzzi & Bizzozero 1977, Rand & Munden 1992). The ECPs of *A. invadans* probably carry out a similar function. It is also possible that some of the ECPs are involved directly in the suppression of elements of the host immune system, such the macrophage respiratory burst which was reduced when macrophages were cultured with germlings (Chapter 5). Although it was not certain that the reduced superoxide production was a result of active suppression by *A. invadans*, it is possible that the effect may be a function of the secretions apparently passing from hyphae to macrophages reported by Thompson *et al.* (1999).

In spite of their extracellular enzymes, most oomycetes are rapidly contained and destroyed when injected into fish (Chinabut 1989, Noga 1993a, Lilley & Roberts 1997), which implies that enzymes alone are not sufficient to allow survival in living tissue. If so, *A. invadans* may operate immunosuppression mechanisms similar to those of bacterial fish pathogens such as *Yersinia ruckeri* (Stave *et al.* 1987).

Even in resistant common carp, the early stages of inflammation were unable to prevent the growth of *A. invadans* after injection challenge (Wada *et al.* 1996), so it is unlikely that similar stages of susceptible species are any more effective. This expectation is borne out by the fact that the prevalence of EUS in snakeheads was not reduced by immunostimulation or passive immunisation, although both treatments reduced mortality after challenge (Chapter 6, Chapter 7).

The poor efficacy of ungranulated snakehead macrophages was further reflected by the fact that they were unable to inhibit the germination or growth of *A. invadans* *in vitro* (Chapter 5). On the other hand, silver barb macrophages were able to inhibit both germination and growth, and their mortality was reduced by immunostimulation in spite of the fact that no increase in granulation was observed (Chapter 6). It is possible that the acute inflammatory response of silver barbs is more effective than that of snakeheads, although silver barbs are highly susceptible to EUS (Tonguthai 1985, Vishwanath *et al.* 1997, Lilley *et al.* in press).

It is not clear how the granulocytes interact with *A. invadans* in the early stages of the immune response. The respiratory burst was reduced by the presence of *A. invadans*, especially in the highly susceptible snakeheads (Chapter 5). It is possible that the reduction is due to the macrophages utilising their energy in a different way, such as by releasing lysozyme (Murray & Fletcher 1976). However, it is more likely that the reduction is due to active inhibition of respiratory burst activity by *A. invadans*, in a similar manner to that involved in the pathogenicity of *Phytophthora infestans* to potato plants (Doke 1983).

8.1.3 Granuloma Formation

The enclosure of the hyphae by granulomata in advanced *A. invadans* infections is so commonly observed that it is incorporated in the case definition of EUS (Roberts *et al.* 1994) and was reflected in the original name of EUS, mycotic granulomatosis (Hatai & Egusa 1978). Wada *et al.* (1996) found that resistant common carp formed granulomata in the early stages of the infection and appeared to be able to contain it. Similarly, Chinabut *et al.* (1995) found that striped snakeheads formed granulomata rapidly at temperatures higher than those at which EUS outbreaks occur, and showed immunity to

A. invadans, further implying that the ability to produce granulomata enables fish to contain *A. invadans*.

The present thesis introduced further evidence for the value of granuloma formation by showing that immunostimulation accelerated granuloma formation and reduced mortality after injection challenge (Chapter 6). Even in stimulated fish, granulomata did not form immediately after the challenge in spite of the fact that wound healing and inflammation has been reported to be extraordinarily rapid in striped snakeheads (Chinabut 1989). It is possible that one of the functions of the ECPs of *A. invadans* is to delay the aggregation of granulocytes into granulomata, allowing the hyphae to penetrate beyond the containing granulomatous area.

As granulomata are unlikely to form fast enough to prevent the germination of cysts, their main function is probably containment. This is illustrated by the fact that even in resistant common carp, injected cysts germinated before they were contained (Wada *et al.* 1996), and the fact that reductions in mortality did not appear to stem from prevention of germination (Chapter 6, Chapter 7). However, containment alone is probably sufficient to enable the fish to control the infection, as Chinabut *et al.* (1995) reported that snakeheads recovered from injection challenges if they were placed in high water temperatures to accelerate granuloma formation, even though the temperature was still within the tolerance range of *A. invadans*.

Some of the mechanisms of containment were elucidated by IHC with MAb 3gJC9 (Chapter 4). The putative early stage hyphae, which appeared to be exploratory, were not found where granulomata had formed. Similarly, granulomata prevented the ECP from diffusing through the tissue as in necrotic or inflamed tissue. These observations suggest that the granulomata confine the means by which the infection can

spread through the fish, and probably the ability to obtain nutrients by extracellular digestion.

The prevention of the diffusion of the ECP through tissue appeared to be very effective where the granulomata had developed, and the mycelium probably needed to grow faster than granulomata were able to form around it to survive. This race between the mycelium and the fish immune system may explain why EUS is at its most destructive during the colder seasons (Tonguthai 1985, Chinabut 1998), as T cells that produce cytokines promoting the development of the immune response are suppressed at low temperatures in many species (Bly & Clem 1992). The importance of the speed of the response may explain why some species are vulnerable to EUS in spite of their ability to increase granulocyte production in response to infection with *A. invadans* (Cruz-Lacierda & Shariff 1994, Pathiratne & Rajapakshe 1998) and form granulomata (Wada *et al.* 1994, Chinabut *et al.* 1995) at low temperatures.

However, the present thesis found that immunostimulation improved the survival of barbs after challenge, although granuloma formation was not enhanced (Chapter 6). Containment may be less important to barbs than snakeheads, as the respiratory burst of barbs was not significantly reduced by *A. invadans* while that of snakeheads was (Chapter 5).

Granulomata are associated with many hyphal infections of fish such as exophialiasis (Pedersen & Langvad 1989) and paecilomycosis (Lightner *et al.* 1988). However, their absence has been noted in the case of one of the few other invasive oomycete fish pathogens, the *Saprolegnia* sp. that causes winter saprolegniasis in channel catfish (Bly *et al.* 1992). It is likely that the *Saprolegnia* sp. that causes winter saprolegniasis is as vulnerable to the granulomatous response as *A. invadans* appears to be. The fact that winter saprolegniasis outbreaks occur after cold snaps (Bly *et al.*

1992), when the hosts are immunosuppressed, may be due to the fact that the pathogen is only able to infect when the granulomatous response is inhibited (Bly *et al.* 1993a).

If the ECPs contain enzymes, their containment may not only prevent damage to the host tissues, but deny the hyphae the products of extracellular digestion. If so, the granulomata are effecting a form of what Alexander & Ingrams (1992) described as nutritional immunity. Nutritional immunity involves immune factors combating pathogens by denying them essential nutrients, and has only been attributed to humoral factors such as interferons and transferrin. López-Dóriga & Martínez (1998) found that cells enclosing pathogenic *Saprolegnia* sp. in brown trout were more characteristic of endothelial cells than leukocytes, and it was not clear how they were interacting with the hyphae. Endothelial cells may have been controlling the infection simply by containing extracellular digestion, without any direct interaction with the hyphae.

The fact that resistance to EUS is so closely linked with the speed at which granulomata form suggests that the migration speed of granulocytes may be more important than their activity. Tilapia granulomata have been shown to form extremely quickly in response to mycobacteriosis (Wolf & Smith 1999), which may be relevant to their resistance to *A. invadans* (Khan *et al.* 1998).

8.2 The Non-Specific Humoral Immune Response

In spite of the importance of complement in the response of mammals to hyphal pathogens (Lehmann 1985), the role of serum factors in the immunity of fish to hyphal pathogens has received very little attention. The few studies that have been carried out have been on *Saprolegnia* spp. (Mulcahy 1969, Mulcahy 1971, Richards & Pickering 1979, Durán *et al.* 1987, Szalai *et al.* 1994).

Studies of the immune response to EUS have concentrated on acquired humoral immunity (Section 8.3) or the more visible cellular immune response (Section 8.1). The only studies to consider the effect of EUS on serum found considerable reduction in serum protein and haematocrit, but did not consider humoral immunity (Cruz-Lacierda & Shariff 1994, Pathiratne & Rajapakshe 1998)

8.2.1 Complement

Although most of the work on complement has been carried out on mammals rather than fish, the elements of the system that have been closely examined in fish are essentially identical, which probably indicates a similar mode of activity (Nonaka *et al.* 1984, Ji *et al.* 1997).

The complement system is comprised of proteins that are usually dissolved in the blood plasma, but which precipitate out to attach to pathogens when they are activated by target molecules on the pathogen surface by the so-called alternative complement pathway (ACP). Also, complement may be activated by antibodies (Section 8.3) in the so-called classical pathway, or by acute phase proteins (Section 8.2.2) bound to the pathogen (Alexander & Ingram 1992, Yano 1996). Fish typically show far higher ACP activity than mammals, implying that the ACP is more important in fish (Matsuyama *et al.* 1988, Yano 1990).

Studies on hyphal infections of mammals indicated that ‘all fungi studied so far have been capable of activating the alternative pathway of complement’ (Lehmann 1985). However, none of the fungi were oomycetes, and Kozel (1996) suggested that the alternative pathway was activated by chitin, which is not present in oomycetes.

The activation of complement did not always confer protection. The opportunist human pathogens, *Aspergillus flavus* and *A. fumigatus*, cleave the complement proteins and so protect themselves (Washburn *et al.* 1986, Sturtevant & Latgé 1992), and Kozel

(1996) suggested that once the chitinous cell wall had formed, complement alone was unable to damage it. One exception was the inhibition of the growth of the dermatophyte *Trichophyton rubrum*, where complement interfered with the construction of the cell wall (Dahl & Carpenter 1986). The present thesis revealed inhibition of germination and growth by a heat-labile factor, presumed to be complement, in several species (Chapter 5). *A. invadans* may be more vulnerable to complement compared to the aforementioned fungi due to its lack of chitin.

Interactions between complement and hyphal pathogens of fish have not received much attention, although complement deficiencies have been associated with outbreaks of winter saprolegniasis in channel catfish (Hayman *et al.* 1992). However, many parameters of the immune system are suppressed by the cold snaps that precede such outbreaks (Section 1.4.3), so it cannot be assumed that the drop in complement levels alone is critical in rendering the fish susceptible to the disease.

In the present study, the ability of sera to inhibit the germination of *A. invadans* *in vitro* was reduced considerably when complement was inactivated (Chapter 5). The same effect was observed in four of the five species examined, and the fifth, the swamp eel, was taxonomically very different from the others. In several cases, heated sera actually increased the number of cysts that germinated, implying that complement may be the only serum factor to be able to inhibit germination in those cases. It was not clear whether the increase in germination in the presence of serum was due to the cysts responding to the presence of fish tissue, or simply to the serum enhancing the nutritional value of the culture medium. Heat-labile humoral factors also inhibited growth in three species, including the swamp eel, so complement's importance in immunity is likely to persist throughout the infection. These effects were observed in

fish with very low anti-*A. invadans* antibody levels, so activation was almost certainly by the alternative pathway.

The main role of complement in immunity to fungal pathogens of mammals is effected by interaction with granulocytes. Enhancement of granulocyte chemotaxis by complement bound to human pathogens including *Candida albicans* (Diamond *et al.* 1978) and *Rhizopus oryzae* (Chinn & Diamond 1982) has been observed. Once the macrophages attached to *T. rubrum*, the presence of complement increased their respiratory burst by over 80% (Dahl & Carpenter 1986).

Studies *in vitro* found that complement opsonised *R. oryzae*, so that the inhibition caused by serum and granulocytes together was greater than that of either alone (Chinn & Diamond 1982). It is possible that complement may opsonise *A. invadans* for fish granulocytes, though this interaction would not have been revealed in the present study as the inhibitory activity of serum and macrophages together was not assessed.

Although the ability of serum complement to inhibit growth is probably important in immunity to *A. invadans*, its ability to inhibit germination may be less relevant as germination probably takes place on or in the dermis, where serum is not present. However, complement is also expressed externally in the mucus (Shephard 1994), which is probably present during germination. Mucus is involved in defence against saprolegniasis in several species (Willoughby & Pickering 1977, Wood *et al.* 1988), so it probably has a function in defence against *A. invadans* as well.

8.2.2 Other Serum Factors

In addition to complement, fish have many other serum factors which confer immunity to a wide range of pathogens, such as lysins that rupture cell membranes, lectins that agglutinate some pathogens, and transferrin that chelates iron and denies it to pathogens

(Alexander & Ingram 1992). Several lectins have been identified in snakeheads *Channa* spp, that are probably involved in immunity (Manihar & Das 1990, Manihar *et al.* 1990, Manihar *et al.* 1991). It is not known which of the many humoral factors are involved in defence against hyphal infections, although an unidentified factor of serum of EUS-resistant common carp was found to inhibit *A. invadans* after complement had been inactivated (Kurata *et al.* 2000b).

Mulcahy (1969, 1971) found that the concentration of some proteins in the serum of salmonids infected with saprolegniasis increased, in spite of a general decrease in serum protein concentrations. Later studies on brown trout found that the activity of some digestive enzymes was considerably increased by *Saprolegnia* infection (Durán *et al.* 1987). Although it is not clear whether the two studies are describing the same proteins, it can evidently not be assumed that the former finding was the result of an immune response.

Although most of the inhibition of germination of *A. invadans* appears to be attributable to complement or antibodies, some other factors were evidently involved in the inhibition of growth (Chapter 5). Growth was inhibited by heated serum of gourami in spite of a very low RAC. It is unlikely that antibodies were involved in the case of the silver barb, as their heated serum did not inhibit germination, while antibody-rich high temperature snakehead serum inhibited germination but not growth.

There was also a heat-labile factor in swamp eel serum that inhibited growth but not germination. The fact that this factor was unable to inhibit germination suggests that it was not complement, as heat-labile factors that inhibited growth in other species always appeared to inhibit germination as well. The considerable taxonomic, physiological and anatomical differences between swamp eels and the other species in the study, and the paucity of information on any aspect of synbranchid physiology,

make it possible that swamp eels may possess immune factors not present in the other species.

What innate humoral factors are involved in the immunity of fish to hyphal pathogens remains a matter for conjecture. Iron deficiency is a problem faced by many invasive pathogens, and host immune systems exploit it by infusing infective sites with transferrin to make iron unavailable to the pathogen (Syed *et al.* 1979), though this has not been studied in hyphal infections.

Rises in C-reactive protein levels have been recorded in humans infected with *Candida albicans* (Kostiala & Kostiala 1987). C-reactive protein is one of the acute phase proteins, so-called because they increase in concentration rapidly in the early stages of infections (Ramos & Smith 1978). Levels of a very similar acute phase protein, phosphorylcholine reactive protein (PRP), were reduced considerably by cold shock in channel catfish. Low levels of PRP was suggested as a possible mechanism by which cold shocked channel catfish are rendered susceptible to winter saprolegniasis, although interactions between PRP and the pathogen have not been examined (Szalai *et al.* 1994).

8.3 Acquired Immunity

Mammals acquire immunity to specific pathogens by both cellular and humoral mechanisms. Although cells that appear analogous to those involved in the adaptive cellular immune response of mammals have been identified in fish, it is not clear whether they function in the same way (Manning & Nakanishi 1996, Warr 1997). Consequently, the present thesis concentrated on the adaptive humoral response, which is mediated by antibodies.

Antibodies are comprised of immunoglobulins (Igs), which are four-chain glycoproteins, with three binding sites. Two of the sites bind foreign molecules, such as those found on pathogen surfaces. The other binds other elements of the immune system to enable them to attack the pathogen. Fish Igs are usually bound into tetramers. Typical methods of inhibition include assisting the attachment of macrophages or complement (Elcombe *et al.* 1985, Kaattari & Piganelli 1996, Yin *et al.* 1996).

8.3.1 Antibody-Mediated Immunity to EUS

Antibody-mediated immunity to hyphal infection is well known in diseases of humans such as aspergillomycosis and candidiasis (Schønheyder & Andersen 1983, Kostiala & Kostiala 1987, Casadevall 1995). Antibody responses to hyphal pathogens of fish have received considerably less attention, although the antibody response to bacterial and viral pathogens is well documented (Kaattari & Piganelli 1996, Warr 1997).

Precipitating antibodies to *Saprolegnia* sp. were found in Atlantic salmon, but the specificity of the response was not considered, and no *Saprolegnia*-naïve fish were sampled, so it is unclear whether the antibodies were produced in response to the pathogen or present as part of a non-specific response (Hodkinson & Hunter 1970). Conversely, Sohnle & Chusid (1983) found no precipitating antibody activity in rainbow trout before or after challenge with various saprolegniaceans, although none of their isolates proved to be pathogenic.

Studies of the response to EUS found that both striped snakeheads (Thompson *et al.* 1997) and rainbow trout (Thompson *et al.* 1999) developed anti-*A. invadans* antibodies after injection challenges, though functional aspects of the immune response were not examined.

The present study found that the presence of antibodies in striped snakeheads inhibited the germination of *A. invadans in vitro* (Chapter 5), and the level of inhibition

was indicative of the level of protection (Chapter 7). It was not established how the antibodies interacted with *A. invadans*, but inactivated serum from snakeheads with high RACs inhibited germination (Chapter 5), indicating that at least some of the antibodies present inhibit *A. invadans* independently of other elements of the immune system. Fish antibodies have been observed to inhibit pathogens by blocking the activity of their antigens (Bricknell *et al.* 1997), or by agglutinating or precipitating them (Yin *et al.* 1996). Any or all of these may have been taking place.

However, the RACs did not have a direct relationship with inhibitory activity within sample groups (Chapter 7), which implies that at least some of them were being limited by the concentration of another factor. One of the more likely mechanisms by which antibodies may have been operating was by facilitating the binding of complement, which is important in the defence of humans to *Aspergillus fumigatus* (Schønheyder & Andersen 1983). The importance of complement has already been described (Section 8.2.1), so a mechanism that improves its binding would be of considerable defensive value.

Antibodies also act as opsonins, enhancing the binding of granulocytes to pathogens (Yin *et al.* 1996). It is not clear whether anti-*A. invadans* antibodies interact with granulocytes as they were always considered separately in the studies *in vitro*. Enhanced antibody production coincided with an enhanced granular response and improved survival in fish fed with Salar-bec (Chapter 6), but it was not established whether the antibodies and granulocytes were interacting. Conversely, enhanced survival conferred by anti-*A. invadans* antibodies could not be associated with improved cellular immunity (Chapter 7), although the small sample sizes and irregular sampling regime were far from ideal for monitoring the development of the inflammatory response in that trial.

8.3.2 Expression of Anti-*A. invadans* Antibodies

The finding that EUS-naïve snakeheads had a measurable anti-*A. invadans* antibody response (Chapter 7) must be considered when evaluating the results of other immunological studies. The presence of non-specific activity is not unprecedented, as mammals may develop non-specific immunity to pathogens through exposure to related non-pathogenic microbes that share antigens (Stainsby *et al.* 1989). Saprophytic *Aphanomyces* spp. are extremely common in fresh water bodies (Srivastava 1980) and many antigens are shared within the genus (Petersen *et al.* 1996, Lilley *et al.* 1997c, Chapter 4). Further evidence of the cross-reactivity of antibodies raised to *Aphanomyces* spp. may be drawn from the fact that even strongly inhibitory antibody responses, almost certainly raised in response to *A. invadans*, cross-reacted with non-pathogenic *Aphanomyces* spp. Such non-specific activity is not restricted to snakeheads, as EUS-naïve rainbow trout have also been found to have measurable anti-*A. invadans* antibody titres (Thompson *et al.* 1999).

However, changes in the nature of the antibody response evidently occurred after exposure to EUS, as sera from EUS-naïve fish showed little or no inhibitory activity and conferred no protection. Snakehead sera from areas where EUS had only recently been introduced showed much higher RACs, although the inhibitory and protective activity of the serum was not much higher than that of naïve fish. In sera from areas that had been exposed for a number of years, RACs had returned to similar levels as found in naïve fish, although inhibitory and protective activities were much higher (Chapter 7).

Improvements in fish antibody responses have been recorded before, although Arkoosh & Kaattari (1991) concluded that increased activity was effected simply by increased antibody concentrations. The differences in the antibody responses of

different snakehead populations (Chapter 7) are evidently not a result of enhanced antibody production alone, though whether they are caused by changes in the antibody's interactions with the antigens or effector mechanisms (Section 8.3.1) was not established.

The increase in the efficacy of the antibody response may be responsible for the reduction of the length and severity of successive outbreaks in the same region (Chinabut 1998). However, if the antibody response is a major factor in the epidemiology of EUS (Section 1.1.4), it is not clear why there are periodic resurgences of EUS outbreaks (Chinabut 1998). A similar pattern has been reported in the case of UM (Blazer *et al.* 1999, Kane *et al.* 2000), and appears to be characteristic of *A. invadans*.

Snakeheads from central Thailand have proved to be susceptible to challenge by injection (Roberts *et al.* 1993, Chinabut *et al.* 1995, Lilley & Roberts 1997, Chapter 6) and even immersion (Fairweather 1999), in spite of their long term exposure to *A. invadans* and consequent antibody response (Chapter 7). The antibody response is evidently unable to provide complete protection even when fully developed. Antibody production in many fish is reduced by stress (Mazur & Iwama 1993, Nagae *et al.* 1994, Einarisdóttir *et al.* 2000), so it is possible that resurgences are due to fish being exposed to unusual levels of environmental stress, which is a major risk factor for EUS (Section 1.1.4).

Low temperature is often associated with EUS, and the present study found that snakeheads kept at low temperatures had a reduced RAC, which was reflected in the inhibition of germination by the serum when complement was inactivated (Chapter 5). Low temperature has been directly linked to low antibody production in striped bass (Carlson *et al.* 1995), so it is likely that the protection conferred by the acquired

immune response may not be at its most effective at the low temperatures at which EUS occurs.

The pattern was far less clear in wild populations, as serum was actually more inhibitory during the winter in one case (Chapter 7), though this may reflect different levels of exposure to *A. invadans* or even other oomycetes that were not a factor in the study of fish kept in an aquarium (Chapter 5).

Like complement, antibodies are expressed in the mucus (Shephard 1994), which is the first point of contact for *A. invadans* zoospores. It is not clear whether antibodies injected intraperitoneally would have passed to the mucus, so this aspect of immunity may not have been induced by intraperitoneal injection of immune serum. However, Wilson (1976) discussed the possibility of immunity to hyphal pathogens being conferred through mucus antibodies, and such a mechanism may be used by snakeheads against EUS.

In ornamental fish tanks, resistance has been transmitted from fish that had recovered from ichthyophthiriasis to fish that had not been exposed, even when the fish were of different species (Ling *et al.* 1991). The transfer of resistance was attributed to antibodies leached from the mucus of the recovered fish. As a method of conferring resistance, this is probably limited to systems that combine high stocking densities with little or no through-flow of water. It is possible that it may be useful against EUS in ornamental fish culture, though the management practices necessary to identify resistant and naïve fish probably preclude its application.

It was not established whether the pattern of antibody mediated immunity found in snakeheads is typical of EUS-susceptible species. Tilapia and gourami from an EUS-endemic region did not have a strong antibody response (Chapter 5), though their exposure to EUS was not known. Rainbow trout injected with *A. invadans* have been

observed to develop an antibody response (Thompson *et al.* 1999), showing that the capacity to do so is not restricted to snakeheads. The fact that the decrease in prevalence and severity of successive outbreaks is observed in all susceptible species (Chinabut 1998) gives strong circumstantial evidence for the response observed in snakeheads being typical, but further studies would be required to establish whether that is the case.

8.4 Monitoring of EUS

As with most fish pathogens, the clinical signs of EUS are not characteristic and are shared by many other diseases (OIE 1997). The case definition of invasive hyphae can only be confirmed by histopathological examination (Roberts *et al.* 1994, Section 1.1.5).

At present, two histopathological methods are routinely used. Grocott's (1955) methenamine silver (GMS) stains the muco-polysaccharides around the hyphae and has been used by the majority of studies of the histopathology of EUS (Roberts *et al.* 1993, Wada *et al.* 1994, Wada *et al.* 1996, Viswanath *et al.* 1997). More recently, the Uvitex stain for cellulose (Wachsmuth 1988) has been adapted for the detection of *A. invadans* (Yorisada *et al.* 1999). The present study considered two other possible diagnostic techniques, namely IHC using MAbs and immunoprevalence monitoring.

8.4.1 Histopathology

GMS clearly identifies hyphae, but it involves expensive reagents and is not specific to *A. invadans*, so an experienced pathologist is needed to distinguish EUS from other hyphal infections by opportunistic saprophytes. A further disadvantage was implied when Viswanath *et al.* (1997) found that it did not stain hyphae in sections that otherwise showed all the appearance of an early stage of EUS, although they attributed the pathology to *A. invadans*. Comparison of GMS and IHC with MAb 3gJC9 showed

that GMS does not stain the earliest stage of the hyphae to penetrate below the dermis, so an early stage infection with *A. invadans* may be overlooked by a pathologist relying on GMS.

Uvitex has only recently been adapted for the study of EUS (Yorisada *et al.* 1999), and there are no reports of detailed histopathological studies with it. As it stains cellulose, it may stain the early stage of the mycelium that is not detected by GMS. Uvitex is less expensive and more rapid than either GMS or IHC, so it has considerable potential for routine monitoring. As it reacts to cellulose, its activity may be limited to oomycetes, which would make it more specific than GMS, though less so than MAb 3gJC9. Further research is required to compare Uvitex with IHC.

Previous immunocytochemical studies of the genus *Aphanomyces* have found considerable antigenic homogeneity (Petersen *et al.* 1996, Lilley *et al.* 1997c), and many MAbs raised to oomycetes have not been species or even genus specific (Estrada-Garcia *et al.* 1989, Burr & Beakes 1994, Hardham *et al.* 1994, Bullis *et al.* 1996). In spite of the fact that mice in the present study were immunised with whole germlings, MAb 3gJC9, which was later used for IHC, only cross-reacted with *A. astaci*. As *A. astaci* does not infect fish (Lilley & Roberts 1997), MAb 3gJC9 is effectively specific for *A. invadans* in fish tissue. Its specificity enabled the recognition of *A. invadans* hyphae that had not penetrated beyond the dermis, and so were not identified when stained by GMS. If the hyphae had been stained with Uvitex, a similar erroneous conclusion would have been drawn.

The specificity and sensitivity of MAb 3gJC9 strongly recommends it for use in assessing experimental challenges. However, it is more expensive and time consuming than Uvitex, so it may be less desirable for routine monitoring.

The ECP of *A. invadans* recognised by MAb 3gJC9 is not expressed by any of the non-invasive oomycetes tested. It is possible that such a characteristic antigen may be involved in the characteristic invasive behaviour of *A. invadans* and *A. astaci*, although no direct evidence was found from the present study.

8.4.2 Immunoprevalence

A major disadvantage of histopathological monitoring of disease is that infected fish must be caught and processed to determine the presence of the disease. Consequently, it is necessary to sample large numbers of fish to determine the absence of EUS (Martin *et al.* 1987), while its presence cannot be established until an outbreak has already had time to cause considerable losses (Shariff 1997).

Immunoprevalence monitoring utilises the presence of antibodies to a pathogen within a population to establish the presence or absence of the pathogen. Its potential has not been widely explored in fish, although it has been recommended as a way of tracking the spread of *Aeromonas salmonicida* in salmonid populations (Yoshimizu *et al.* 1992). Mulcahy (1969) suggested that saprolegniasis could be diagnosed by changes in the serum proteins, but the nature of the proteins that changed in concentration was never established, and the suggestion was never followed up.

The presence of anti-*A. invadans* antibodies in EUS-naïve fish and the low RACs after long term exposure (Chapter 7) make it unlikely that immunoprevalence has much potential for routine monitoring of the distribution of *A. invadans*.

However, the sudden increase in RAC during EUS outbreaks in previously unexposed populations suggests a possible application. Examination of snakehead sera may be recommended when wild fish are observed to show the clinical signs of EUS in a region where it had not previously occurred. In such a case, an ELISA of the sera of 10-20 individuals should allow fisheries inspectors to establish whether or not the

disease is EUS without the need to catch individuals actually showing clinical signs. The particular advantage of this is that wild-caught snakeheads are often sold alive at local markets where they are available to fisheries officers with limited resources. As fish with lesions are not usually marketable (Palisoc & Aralar 1990, Bhaumik *et al.* 1991, Lilley *et al.* in press), fisheries officers have to catch fish for histopathological examination themselves, which presents many logistical difficulties.

It is also conceivable that the high inhibitory activity of snakehead serum after long term exposure to EUS may be used to indicate endemic regions. However, fungicidal assays are not a standard protocol in most diagnostic laboratories, so this technique is unlikely to see wide scale application. A further difficulty is that as *A. invadans* itself must be used in such assays, it would only be advisable where facilities and working conditions are able to prevent accidental releases, which could not be guaranteed by many regional fisheries offices in Asia.

8.5 Control of EUS

Most recommendations for the control of EUS have centred around management strategies or water treatment (Section 1.1.6). Aquaculture disease control strategies in Europe and North America often involve enhancing the immune system of the fish to make it more able to fight off infections by vaccination or immunostimulation.

Vaccination involves exposing the fish to the antigens of specific pathogens in order to induce an antibody response to specific pathogens (Ellis 1988a). Immunostimulation provokes a more general increase in the activity of the fish immune system (Galeotti 1998, Sakai 1999).

8.5.1 Immunostimulation

A wide range of dietary supplements are available for use in aquaculture, although they are mainly used in intensive systems that depend on large financial investments (Sakai 1999). However, the feed supplement ascogen has been recommended for use in the farming of tilapia (Ramadan *et al.* 1994) and silver barbs (Kularatne *et al.* 1994), both of which are usually cultured in low input systems.

Immunostimulation has been recommended for situations in European aquaculture where poor health stems from stress (Raa 1996) or non-specific pathogens (Vadstein 1997). The fact that susceptibility to EUS is related to stress (Sections 1.1.4, 8.3.2), and the prevalence of non-specific pathogens in tropical pond farms (Section 1.1.2), suggest that the recommendations may be relevant in the control of EUS, and indeed other diseases in Asian aquaculture.

The value of Ergosan was unclear from the present study due to the difficulties encountered in the feed trial (Chapter 6). It reduced the mortality of snakeheads, which suggests that it reduced their susceptibility to stress, implying that it may reduce susceptibility to EUS even though it was not clear whether mortalities were due to EUS in that particular study. It is possible that the mortalities were caused by opportunists such as *Aeromonas hydrophila*, which is one of the major diseases in south east Asian aquaculture in spite of its non-specific nature (Lio-Po *et al.* 1992, 1998, Torres *et al.* 1993). Whatever the cause, the fact that mortality was reduced indicates that Ergosan may have potential for use in freshwater Asian aquaculture, and warrants further research.

The aquarium trial with the vitamin supplement, Salar-bec, indicated an unambiguous improvement in the resistance of fish to EUS. The inflammatory and granulomatous responses (Section 8.1.3) were accelerated and antibody production

(Section 8.3.2) was enhanced. Both of these factors are involved in defence against *A. invadans*, and coincided with a reduction in mortality. There is considerable evidence for the value of vitamin supplements as immunostimulants (Sakai 1999), so these results are not unprecedented.

Striped snakeheads on a vitamin-poor diet heal slowly and show poor development of the granulomatous response (Chinabut 1989). Similar results were found in Nile tilapia (Jauncey *et al.* 1985), although that has little relevance to EUS. Snakeheads fed on a Salar-bec supplemented diet showed an immune response more characteristic of fish kept at higher temperature (Chinabut *et al.* 1995), in a manner similar to channel catfish fed on a vitamin C-supplemented diet and challenged with edwardsiellosis (Durve & Lovell 1982). Temperature mediated effects on the inflammatory response of silver barbs have not been described, but Salar-bec increased the inflammation and reduced mortality, so it probably had a similar effect on silver barbs to that on snakeheads.

One situation where immunostimulants may be of particular interest is in cage farming. EUS has been identified as one of the major constraints to cage aquaculture in Bangladesh (McAndrew in press), and it is not possible to apply many of the successful management strategies (Lilley *et al.* 1998) or water treatments (Campbell *et al.* 2001) to uncontained bodies of water.

While the aquarium trials indicate the potential of the immunostimulants (Chapter 6), it cannot be assumed that the controlled conditions required for aquarium trials are replicated on farm systems (Galeotti 1998). This problem was apparent in a previous attempt to evaluate Ergosan on a salmon farm, where the results were inconclusive in spite of the potential that Ergosan had shown in preliminary studies (Hall 1998). The diversity of species, systems and feeds used in Asian aquaculture

(Tavarutmaneekul *et al.* 1996) make generalisations about the value of any alteration in the feeding regime even more inappropriate than they are in European or North American aquaculture. Before Salar-bec can be recommended for use on any farm system, it will have to be assessed, and the dose rate optimised, for use on that system.

8.5.2 Vaccination

The improvement in immunity of fish to specific pathogens following exposure to killed or attenuated preparations of them has been known for nearly 60 years (Duff 1942). Subsequent studies have shown that antigens may be prepared in a form that induces a protective antibody response in fish to many bacterial (Anderson & Nelson 1974, Baba *et al.* 1988, Nikl *et al.* 1991) and viral (Amend & Smith 1974, Nishimura *et al.* 1985) pathogens. There have been far fewer reports of the potential of vaccines against hyphal pathogens of fish, although mammals have been successfully vaccinated against hyphal pathogens (Gudding & Naess 1986, Mendoza *et al.* 1992b, Pier & Zancanella 1993).

The only attempts to induce an immune response against hyphal pathogens in fish have been carried out using *A. invadans*. Snakeheads injected with homogenised zoospores or mycelium developed antibodies to them (Thompson *et al.* 1997), though it was not established whether those antibodies were protective. Conversely, rainbow trout only developed antibodies to zoospores and not to mycelium (Thompson *et al.* 1999). The antibody response of snakeheads (Thompson *et al.* 1997, Chapter 5, Chapter 6, Chapter 7) suggests that it may be possible to induce a protective antibody response in farmed snakeheads.

Although vaccination is widely used in European and North American aquaculture (Ellis 1995), it may be prohibitively expensive for less intensive, lower input systems such as snakehead farms. A further concern is that fish are not handled

between stocking and harvest in most Asian systems. Vaccination may not be appropriate at the time of stocking as the ontogeny of the adaptive immune system of many species is not complete (Ellis 1988*b*, Tatner 1996). Handling has been found to make farmed salmon more vulnerable to opportunistic infections (Strasdin & McBride 1979), and the many opportunists present in most tropical pond or cage farms make it inadvisable to handle fish for the sake of a vaccine that would only confer protection against one disease. Opportunists are likely to be a far greater problem in the poorer quality waters found in Asian pond farms when compared to salmonid farms, and several are known to be extremely common (Section 1.1.2).

A further concern is that the different responses to vaccination of snakeheads and trout (Thompson *et al.* 1997, 1999) imply that the response to vaccination varies between species. Even the possibility of differences necessitates the optimisation of any vaccine for every species before it could be used for them, which would further add to the cost and the time needed before the vaccine could be applied to farm systems.

8.6 Recommendations for Future Research

8.6.1 Applications of Monoclonal Antibodies

A viable diagnostic method has been developed using MAb 3gJC9, which may be further optimised by application of protocols not examined in this thesis, such as by microwaving during incubation with antibodies to accelerate the reaction (Mayer & Bendayan 2001). Comparison between IHC and Uvitex is necessary to establish whether MAb 3gJC9 has a role in diagnostics.

It is also possible that MAb 3gJC9 could be adapted for diagnosis of crayfish plague.

It should be noted that the cross-reactivity of MAb 3gJC9 has only been examined against other oomycetes and fungi and observed by immunocytochemical and immunohistochemical methods. It is not impossible that it may cross-react with other microbes that may be present in fish showing clinical signs of EUS, though this should not cause confusion as they would be easily distinguished from hyphae in histological preparations.

Several antibodies raised against oomycetes are used for ELISAs or dot-blots to detect plant pathogens in soil or water samples (Ali-Shtayeh *et al.* 1991, Cahill & Hardham 1994). It is possible that a similar assay may be developed with MAb 3gJC9, but its specificity and sensitivity would have to be reassessed for the procedure used.

MAb 3gJC9 could be of considerable value for research into the early stages of infection by *A. invadans* if applied to fish challenged by immersion and sampled sequentially. IFAT and electron microscopy should be used to gather more detail about the early stage of the mycelium reported here, and to observe the penetration of the skin and induction of necrosis by *A. invadans*.

Although the other four MAbs have little potential for diagnostic work due to their lack of specificity (Chapter 4), all five may be useful for future research. Non-specific MAbs raised to oomycetes have been used for studies of taxonomic characteristics (Arashima *et al.* 1994, Beakes *et al.* 1995, Cope *et al.* 1996), life cycle (Hardham & Suzuki 1986, Estrada-Garcia *et al.* 1990) and pathogenicity (Gubler & Hardham 1988), and the MAbs raised in the present study may have similar potential.

8.6.2 Extracellular Products

The ECPs of *A. invadans* have received scant attention in the past but may be important in pathogenicity (Section 8.1). Many of the ECPs are likely to be enzymes (Unestam 1966), so it would be valuable to establish whether any show proteolytic or hydrolytic

activity. It would also be of interest to establish whether the haemagglutinating and haemolytic properties of *A. invadans* (Kurata *et al.* 2000) are expressed extracellularly, as would be expected if they are involved in the process of infection.

The possibility that ECPs are involved in the inhibition of macrophage activity (Chapter 5) could be examined by comparing superoxide production in the presence of ECP with that in its absence. The effect of ECP on macrophage migration could also be examined *in vitro* in order to test the possibility that ECPs inhibit the formation of the granulomatous response.

The MAbs could be used in any or all of these contexts to establish whether the activity of such ECPs is affected by neutralising the antigens recognised by the MAbs.

8.6.3 Non-Specific Immunity to *A. invadans*

Several areas of the non-specific immune system that interacted with *A. invadans* were identified.

The finding that *A. invadans* inhibited the respiratory burst response could be confirmed by comparing the respiratory burst in the presence of *A. invadans* with that in the presence of a non-invasive *Aphanomyces* sp.

The importance of serum factors other than those established in the present study could be assessed by performing a range of assays on complement-inactivated serum and correlating the results with the inhibitory activity of serum. Recommended tests include agglutination and lysozyme assays.

Although the present study described several interactions between the immune system of host species and *A. invadans*, it did not establish why certain species are resistant. Further studies in this area should consider the mucus to establish whether the same inhibitory processes take place in mucus as in serum. It may also be of value to compare the migratory response of macrophages towards *A. invadans in vitro*, and to

compare the activity of macrophages in the presence of serum with the activity of either macrophages or serum alone.

8.6.4 Acquired Immunity to *A. invadans*

The main objective of further research into acquired immunity to *A. invadans* should be to establish whether the patterns observed in striped snakeheads are typical of EUS-susceptible species.

It would also be valuable to establish the method by which antibodies inhibit *A. invadans*, and whether antibodies are expressed in the mucus.

8.6.5 Control of EUS

There may be some potential for vaccine development in more intensively farmed species, where higher investments may justify the expense of vaccination, and hatchery reared seedstock may not have the exposure to *A. invadans* observed in wild-caught snakehead seedstock. However, the developmental, logistical and financial constraints are likely to prove prohibitive, so vaccine development should not be considered a priority.

The main suggestion for control of EUS derived from the present thesis is by application of immunostimulants. At the time of writing, pond trials with Ergosan and Salar-bec are under way at the Central Institute of Freshwater Aquaculture, Bhubaneswar, India. If these trials indicate that one or both immunostimulants is successful in ponds, it is strongly recommended that they should be developed for use on farms.

Application on farms should be preceded by optimisation of dose regimes with due regard for possible deleterious effects on the fish and expense, and it cannot be assumed that optimisation for one species indicates optimisation for any other. Careful

monitoring and comparison of the costs and benefits of application should be implemented when the immunostimulants are first applied on farms, as it is possible that their use would only be financially beneficial on farms when an EUS outbreak is known to be likely.

8.7 Conclusions

8.7.1 The Pathology of EUS

- *A. invadans* releases extracellular products that probably cause much of the necrosis associated with EUS.
- Some of the extracellular products of *A. invadans* are identical to those released by *A. astaci*.
- An aspect of the infection, believed to be an early stage of the mycelium, was identified for the first time.
- *A. invadans* reduces the respiratory burst activity of macrophages.

8.7.2 Non-Specific Immunity to EUS

- Complement is the main aspect of the innate humoral immune response involved in the response against *A. invadans*.
- Macrophage activity appears to be actively inhibited by *A. invadans*.
- Rapid formation of granulomata is necessary to contain the mycelium, and confers protection.
- The speed at which granulomata form can be increased by adding vitamin supplements to feed.

8.7.3 Adaptive Immunity to EUS

- Striped snakeheads exposed to EUS develop a protective antibody response to *A. invadans*.
- Striped snakeheads develop antibodies against *A. invadans* rapidly after their first exposure, but the response takes several EUS seasons before it confers its maximum level of protection.

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Appendix I. Formulae of Solutions

All solutions are prepared in distilled water unless otherwise specified.

Acid Alcohol (H&E)

10% v/v 12M hydrochloric acid (HCl) in methylated spirits.

Antibody buffer (ELISA)

PBS containing 1% w/v Bovine Serum Albumin (BSA)

Autoclaved Pond Water (APW) (Fungus culture)

Natural pond or lake water with pH 6-7 was filtered through Whatman 541 filter paper and autoclaved.

Chloro-naphthol Solution (Dot blot, Western blot)

Prepared in methanol.

0.3% w/v 4-chloro-1-naphthol

5% Chromic acid (GMS)

50g l⁻¹ Chromium trioxide (CrO₃)

Chromogen solution (ELISA)

Substrate buffer is prepared as follows:

21.0 g l⁻¹ Citric acid (C₆H₈O₇)

8.2g l⁻¹ Sodium acetate (C₂H₃O₂Na)

pH 5.4

Substrate is prepared by dissolving 42mM 3'3'5'5'-Tetramethylbenzidine dihydrochloride (TMB) in a solution of 1:2 glacial acetic acid:distilled water.

Immediately before use, chromogen solution was prepared in substrate buffer as follows:

1% v/v Substrate

0.0333% v/v 30% w/w Hydrogen Peroxide (H₂O₂)

Coating solution (ELISA)

1.59g l⁻¹ di- sodium carbonate (Na₂CO₃)

2.93g l⁻¹ Sodium hydrogen carbonate (NaHCO₃)

0.001% w/v Poly-L-lysine

pH 9.6

Developing solution (Western blot)

The following were added to 10ml PBS:

2ml 3.4g l⁻¹ 4-chloro-1-naphthol in methanol

10μl H₂O₂

ELISA blocking solution (ELISA)

3% w/v casein in PBS.

Eosin (H&E)

11% v/v Putt's eosin

89% v/v 1% eosin solution

1% eosin solution is prepared as follows:

10g l⁻¹ eosin yellowish

11 Putt's eosin is prepared by dissolving the following reagents in 100ml ethanol:

10g l⁻¹ eosin yellowish5g l⁻¹ potassium dichromate (K₂Cr₂O₇)

The solution was dissolved in 800ml distilled water and 100ml aqueous picric acid was added.

Fixative Solution (Antigen fixation)

4% v/v formaldehyde in PIPES buffer.

Germedia (Fungus culture)6g l⁻¹ d-glucose2g l⁻¹ Mycological peptone1g l⁻¹ Yeast extract0.256g l⁻¹ Magnesium sulphate heptahydrate (MgSO₄.7H₂O)0.028g l⁻¹ Potassium phosphate (KH₂PO₄)4.8mg l⁻¹ Ferric chloride hexahydrate (FeCl₃.6H₂O)3.6mg l⁻¹ Manganese chloride tetrahydrate (MnCl₂.4H₂O)7.8mg l⁻¹ Copper sulphate pentahydrate (CuSO₄.5H₂O)0.8mg l⁻¹ Zinc sulphate heptahydrate (ZnSO₄.7H₂O)100mM Calcium chloride (CaCl₂.2H₂O)Glucose Peptone Yeast (GPY) broth (Fungus culture)3g l⁻¹ d-glucose1g l⁻¹ Mycological peptone0.5g l⁻¹ Yeast extract0.128g l⁻¹ Magnesium sulphate heptahydrate (MgSO₄.7H₂O)0.014g l⁻¹ Potassium phosphate (KH₂PO₄)0.029g l⁻¹ Calcium chloride dihydrate (CaCl₂.2H₂O)2.4mg l⁻¹ Ferric chloride hexahydrate (FeCl₃.6H₂O)1.8mg l⁻¹ Manganese chloride tetrahydrate (MnCl₂.4H₂O)3.9mg l⁻¹ Copper sulphate pentahydrate (CuSO₄.5H₂O)0.4mg l⁻¹ Zinc sulphate heptahydrate (ZnSO₄.7H₂O)Glucose Peptone Yeast (GPY) agar (Fungus culture)

Prepare GPY broth and add:

12g l⁻¹ Technical agar no. 3Glutaraldehyde solution

PBS containing 0.05% v/v glutaraldehyde.

Glycine Solution (Antigen fixation)

75mM glycine in PIPES buffer.

Hypoxanthine Aminopterin Thymidine media supplement (HAT) (Fusion)

Prepared in DMEM

5mM	Hypoxanthine (C ₅ H ₄ N ₄ O)
0.002mM	Aminopterin (C ₁₉ H ₂₀ N ₈ O ₅)
0.08mM	Thymidine (C ₈ H ₁₈ N ₂ O ₆ S ₂)

Hybridoma maintenance medium (Hybridoma culture)

Prepared in Dulbecco's modification of Eagle's medium.

20mM	L-glutamine (C ₅ H ₁₀ N ₂ O ₃)
1000U ml ⁻¹	Penicillin
1mg ml ⁻¹	Streptomycin
0.5mM	Sodium pyruvate (C ₃ H ₃ O ₃ Na)
20% v/v	Foetal bovine serum heat inactivated at 55°C for 30min.

Immunoblot Developing Solution (Dot blot, Western blot)

Prepared in PBS.

17% v/v	Chloro-naphthol solution
0.0008%	30% w/w Hydrogen peroxide (H ₂ O ₂)

Low Salt Wash buffer (LSW) (ELISA)

2.42 g l ⁻¹	Trizma base (C ₄ H ₁₁ NO ₃)
22.22 g l ⁻¹	Sodium chloride (NaCl)
0.05% v/v	Tween 20
pH 7.3	

Macrophage Isolation Medium (Macrophage culture)

Prepared in L-15 medium.

1% v/v	10,000U ml ⁻¹ Penicillin-streptomycin solution
0.4% v/v	2,500U ml ⁻¹ heparin in L-15
2% v/v	Foetal Bovine Serum (FBS) heat inactivated at 55°C for 30min.
Stored at 4°C.	

Macrophage Washing Medium (Macrophage culture)

Prepared in L-15 medium.

1% v/v	10,000U ml ⁻¹ Penicillin-streptomycin solution
0.1% v/v	Foetal Bovine Serum (FBS) heat inactivated at 55°C for 30min.
Stored at 4°C.	

Macrophage Maintenance Medium (macrophage culture)

Prepared in 2x L-15 medium.

5% v/v	Foetal Bovine Serum (FBS) heat inactivated at 55°C for 30min.
Stored at 4°C.	

Mayer's haematoxylin (H&E)

The following reagents were added and allowed to dissolve overnight.

1g l ⁻¹	Haematoxylin
0.2g l ⁻¹	Sodium iodate (NaIO ₃)
50g l ⁻¹	Aluminium potassium sulphate heptahydrate (AlK(SO ₄) ₂ .12H ₂ O)

The following reagents were added and the solution was boiled for 5min.

1g l ⁻¹	Citric acid (C ₆ H ₈ O ₇)
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50g l⁻¹ Chloral hydrate (C₂H₃Cl₃O₂)

Methyl green solution

PBS containing 0.5% w/v methyl green solution

10% Neutral Buffered Formalin (Histology)

10% v/v 40% formaldehyde (CH₂O)
 4g l⁻¹ Sodium di-hydrogen orthophosphate dihydrate (NaH₂PO₄.2H₂O)
 6g l⁻¹ di-sodium hydrogen orthophosphate (Na₂HPO₄.12H₂O)

Peptone Glucose - 1 (PG-1) agar (Fungus culture)

3g l⁻¹ d-glucose
 6g l⁻¹ Mycological peptone
 12g l⁻¹ Technical agar no.3
 0.17g l⁻¹ Magnesium chloride hexahydrate (MgCl₂.6H₂O)
 0.15g l⁻¹ Calcium chloride dihydrate (CaCl₂.2H₂O)
 0.37g l⁻¹ Potassium chloride (KCl)
 0.02g l⁻¹ Ferric chloride hexahydrate (FeCl₃.6H₂O)
 0.044g l⁻¹ Ethylenediaminetetraacetic acid (EDTA) disodium salt

The glucose was dissolved in 130ml 13mM sodium phosphate buffer and 120ml distilled water. The other ingredients were dissolved in 250ml of distilled water, autoclaved and mixed.

Sodium phosphate buffer was prepared from the following stocks:

Solution A, store at 4°C:

31.2 g l⁻¹ Sodium di-hydrogen orthophosphate dihydrate (NaH₂PO₄.2H₂O)

Solution B, store at room temperature:

71.7g l⁻¹ di-sodium hydrogen orthophosphate (Na₂HPO₄.12H₂O)

407.5ml of solution A, 92.5ml of solution B and 500ml of distilled water are mixed to make 1000ml of sodium phosphate buffer. pH is adjusted to 6.3.

34% Percoll solution (Macrophage culture)

100ml contains:

34ml Percoll
 10ml 10x Minimum Essential Medium
 56ml Autoclaved distilled water

Stored at 4°C.

51% Percoll solution (Macrophage culture)

100ml contains:

51ml Percoll
 10ml 10x Minimum Essential Medium
 39ml Autoclaved distilled water

Stored at 4°C.

Phosphate Buffered Saline (PBS)

0.876g l⁻¹ Sodium dihydrogen orthophosphate dihydrate (NaH₂PO₄.2H₂O)
 2.56g l⁻¹ di-sodium hydrogen orthophosphate (Na₂HPO₄.2H₂O)
 8.77g l⁻¹ Sodium chloride (NaCl)
 pH 7.2

PIPES buffer (antigen fixation)

100mM Piperazine-N,N'-bis[2-ethanesulphonic acid]; 1,4-
Piperazinediethanesulphonic acid (PIPES) ($C_8H_{18}N_2O_6S_2$)
pH 6.8

Reservoir buffer (SDS-PAGE)

15g l⁻¹ Trizma base
72g l⁻¹ Glycine
5g l⁻¹ Sodium dodecyl sulphate (SDS)
pH 8.3

Sample buffer (SDS-PAGE)

The following solutions were prepared and mixed to make 10ml sample buffer:

0.6ml 1M trizma base (pH 6.8)
5ml 50% v/v glycerol
2ml 10% w/v sodium dodecyl sulphate (SDS)
0.5ml 2-mercaptethanol
1ml 1% w/v bromophenol blue
0.9ml distilled water

Scott's tap water substitute (H&E)

Prepared in tap water.

3.5g l⁻¹ Sodium bicarbonate ($NaHCO_3$)
20g l⁻¹ Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)

Silver nitrate working solution (GMS)

50% v/v Methenamine silver nitrate solution
4% v/v 5% borax

The solution is prepared immediately before use. Methenamine silver nitrate solution is prepared as follows:

30g l⁻¹ Methenamine (hexamine) ($(CH_2)_6N_4$)
5% v/v 5% silver nitrate solution

The solution is stored at 4°C in the dark. 5% silver nitrate solution is prepared as follows:

50g l⁻¹ Silver nitrate ($AgNO_3$)
5% Borax is prepared as follows:
50g l⁻¹ Sodium tetraborate (borax) $Na_2B_2O_7 \cdot 10 H_2O$

Transblot buffer (Western blot)

Prepared in distilled water containing 20% v/v methanol.

14.4g l⁻¹ Glycine
3.03g l⁻¹ Trizma base
pH 8.3

Tris buffered saline (TBS)

2.42g l⁻¹ Trizma base ($C_4H_{11}NO_3$)
29.22 g l⁻¹ Sodium chloride (NaCl)
pH 7.5

Triton-supplemented tris buffered saline (TBSX)

TBS containing 1% v/v Triton X-100

Tween supplemented tris buffered saline (TTBS) (ELISA, dotblot, Western blot)

TBS containing 1% v/v Tween 20

Tyramide conjugate (IHC)

The following solutions were incubated in the dark for 2h before dilution in 4.36ml ethanol and storage at 4°C

500µl Tyramine amplification technique (TAT) solution A

1.445µl TAT solution B

TAT solution A is prepared in dimethylformamide as follows:

10mg ml⁻¹ Sulphosuccinimidyl-6-[biotinimide] hexanoate

TAT solution B is prepared in 10mg ml⁻¹ dimethylulformamide as follows:

1.04M triethylamine

V8 broth (Fungus culture)

V8 vegetable juice was centrifuged at 500g for 10min and the supernatant was collected. The following were diluted in distilled water and autoclaved:

5% v/v V8 supernatant

0.2% w/v Calcium carbonate (CaCO₃)

pH 6.1

Wood's extraction buffer (ELISA)

85mM Trizma base

1mM Magnesium chloride (MgCl₂)

1mM EDTA

10mM Potassium chloride (KCl)

0.198g l⁻¹ Ascorbic acid (C₆H₈O₆)

1g l⁻¹ Glycerol (C₃H₈O₃)

5µM Phenylmethylsulphoxylflouride (PMSF)

pH 7.5

Appendix II. Optimised Protocol for Immunohistochemistry of Tissue

Sections Using MAb 3gJC9

All steps in the following protocol are for used on sections that have been prepared as specified in Section 2.2.2, and mounted on glass slides treated with APES as specified in Section 4.2.1.1.

1. Dewax slides by 5min immersions in two changes of xylene, one change of absolute ethanol and one change of 70% ethanol.
2. Wash slides in running water for 10min.
3. Cover sections with 10% goat serum in antibody buffer for 20min.
4. Wash slides by sluicing with TBSX, followed by three 3min incubations in wash buffer.
5. Draw enclosures around sections with a PAP pen.
6. Cover sections with neat MAb 3gJC9 supernatant or unused hybridoma culture medium for negative controls.
7. Incubate slides overnight at 4°C in a humidity chamber.
8. Wash slides as before.
9. Cover sections with 50% v/v FITC-labelled sheep anti-mouse IgG in antibody buffer and incubate for 1h at room temperature in a humidity chamber.
10. Wash slides as before.
11. Repeat steps 4-7.
12. Incubate slides for 1h in a humidity chamber at room temperature.
13. Wash slides as before.
14. Cover sections with methyl green solution for 5min at room temperature.
15. Wash slides in running water for 5min.
16. Remove excess water from slides, and cover sections with DAKO anti-fade fluorescence mounting medium.
17. Immediately apply cover slip and seal edges with nail varnish.
18. Dry and store slides in dark at 4°C until they are viewed.